Quorum Sensing in the Hawai‘ian Coral Pathogen

Vibrio coralliilyticus strain OCN008

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ABSTRACT

Coral disease represents a serious threat to reefs worldwide. Reef ecosystems have been reshaped by coral disease in the Caribbean, Florida Keys, and the Great Barrier Reef. Reefs represent millions of dollars in economic value as well as contribute heavily to marine primary production, thus efforts to minimize such damage have become crucial. Coral disease in Hawai‘i has presented less of a problem when compared to the Caribbean, but emerging diseases over the past decade have brought the potential for similar destruction. One disease of concern in Kāne‘ohe Bay, Oahu, is Acute *Montipora* White Syndrome (aMWS), a rapidly progressing tissue-loss disease affecting the reef-building coral *Montipora capitata.* Early efforts studying this disease identified *Vibrio coralliilyticus* strain OCN008 as an etiological agent of aMWS. Interestingly, OCN008 produces and utilizes the antibiotic andrimid as a novel virulence factor. This discovery represented one of only a handful (<5) of known virulence factors in the field of coral disease, and potentially provides a point at which to begin investigations into preventative and/or curative strategies. This work describes the quorum sensing (cell-density dependent bacterial communication/behavior) mechanics of strain OCN008. The main objectives were the identification and characterization of putative quorum sensing circuits and the role they play in the infection of *M. capitata*. Despite possessing homologs of four known *Vibrio* quorum sensing pathways, OCN008 requires only one, the LuxPQ/S pathway (AI-2), to activate the high cell density response. Quorum sensing also contributes to the virulence of OCN008 at both low and high cell densities. Since the novel virulence factor andrimid was shown here to be under regulation of AI-2 mediated quorum sensing, a scenario is presented in which OCN008 can initiate andrimid production in response to a quorum of AI-2 producing bacteria, regardless of taxonomy. Interestingly, the two most severe outbreaks of aMWS were recorded following

periods of heavy rain, events known to cause increased bacterial abundance and perturbations in coastal microbial communities. Shifts in coral microbiota have been linked to increased incidents of coral disease. This work offers one potential mechanism behind this phenomenon. In addition, this work offers the first direct evidence that quorum sensing is involved in coral disease.

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CHAPTER ONE: INTRODUCTION

Coral disease and its global impact

Coral reefs encompass some of the most diverse habitats on the planet. At their heart lie reef-building corals. Corals provide a plethora of homes to numerous species of fishes, marine reptiles and invertebrates and, of course, countless prokaryotes. It has been estimated that coral reefs provide habitat for up to 2 million different species, representing between one fourth and one third of all marine life[1]. This is remarkable considering that corals cover less than one percent of the ocean floor[2]. Corals, found in the phylum Cnidaria, are colonial organisms that consist of individual animals called polyps: cylindrical bodied organisms topped with tentacles containing nematocysts, which enable capture and digestion of planktonic prey. Reef building corals belong to the order Scleractinia and excrete a calcium carbonate skeleton that creates the reef structure. Corals reproduce sexually by releasing egg and sperm into the water column. Corals typically grow in relatively shallow (>90 feet), warm (70 – 80°F), oligotrophic waters. Corals grow asexually by clonal iteration or 'budding' of new polyps. Growth is dependent on nutrient acquisition, which is achieved not only from predation by polyps, which supply limited amounts of nutrients to the coral, but more importantly by carbon fixation performed by symbiotic dinoflagellates (*Symbiodinium* sp.) that reside in the coral tissue (hence the growth in shallow waters). The symbiotic nature of the coral animal and the *Symbiodinium* allow for sufficient growth to generate large, complex structures that we know as reefs. This process takes years, as coral growth is a slow process ranging from 0.2 to 10 centimeters per year[3,4]. This means that many of the large reefs we know today represent some of the oldest biospheres on the planet. Coral disease has the potential to undo thousands of years of coral production in the

blink of an eye – and thus needs urgently to be better understood in order to prevent such devastating losses.

Incidents of coral disease, first noticed in the 1970s, have been on the steady incline over the last 15 years[5,6,7]. This is alarming as coral reefs provide significant economic benefits in the regions they are found[8]. It is estimated that reefs generate US$ 9.3 billion in tourism, US$ 9 billion in coastline protection and US$ 5.7 billion through their associated fisheries[9]. Roughly 500 million people in 94 countries rely on coral reefs for their livelihood[10,11]. Reef degradation by abiotic factors such as sediment stress, increased sea water temperatures, or human activities such as over fishing and dredging is relatively well understood. However the biotic factors that lead to coral disease outbreaks remain largely elusive. In recent decades, increased rates of outbreaks have made coral disease a major contributor to worldwide degradation[12,13]. Coral disease, loosely defined, is any disorder of structure or function that results in reduced coral health[14]. This includes ailments such as bleaching (the loss of *Symbiodinium,* which, if persistant, leads to colony mortality)[15–17], tumors or growth anomalies that can cause reduction in growth or fecundity[18–21] or tissue loss diseases in which the coral tissue is lysed leading directly to mortality[22–25].

Coral bleaching, characterized by the loss of *Symbiodinium* with the coral tissue intact, poses a significant threat to corals due to the association of bleaching with increased water temperatures. In most species of coral, the *Symbiodinium* provide up to 90% of the carbon requirements of the coral[26]. With sustained absence of the zooxanthellae these corals cannot survive. Increases in water temperatures are expected to continue, thus increasing the chance of mass bleaching events. A sustained increase of 1 to 2 °C above the average summer maxima temperature for a week is often enough to induce bleaching, while 2 periods longer than several

weeks could be catastrophic, resulting in the total loss of coral cover. In addition to abiotic factors, bleaching can be caused by biotic factors such as bacterial pathogens. Strains of *Vibrio shiloi* and *Vibrio patagonia* have been shown to cause bleaching in the coral *Occulina patagonia,* and *Vibrio coralliilyticus* strain BAA-450 has been shown to cause bleaching in the coral *Pocillopora damicornis*[27–29]. Interestingly BAA-450 causes bleaching when water temperatures are between 23-27°C, but when temperatures rise above 27°C tissue lysis is induced, highlighting the importance of understanding the mechanism of virulence associated with increased temperatures. The results of coral bleaching can be massively destructive. In 2015 - 2016 a strong El Nino event caused sustained, elevated water temperatures throughout the Indo-Pacific[30,31]. This devastated corals throughout the region with up to 60-80% bleaching in the worst affected areas of the Great Barrier Reef[32]. In Hawaii, the strong El Nino led event that began in late 2014 led to ~80% of the dominant coral species in Kaneohe Bay, Oahu to bleach[33]. This was by far the largest bleaching event witnessed in the Hawaiian archipelago, likely due to sustained surface temperatures of 30°C, well above average[34].

While coral bleaching is primarily caused by abiotic factors, tissue loss diseases are typically induced by biotic factors (usually pathogens), and pose the most significant and dangerous threat to corals. Tissue loss diseases are, unsurprisingly, characterized by the presence of lesions comprised of lysed coral tissue, with bare skeleton exposed. Affliction with these types of diseases often leads to whole colony mortality at much more rapid rates than bleaching induced mortality (in the 2014 Kaneohe Bay Bleaching event ~90% of tagged colonies that experienced bleaching recovered)[35]. Tissue loss diseases can be problematic, as bare coral skeletons crumbles over time reducing the 3 dimensional structure of the reef[36]. The resulting loose substrate is unsuitable for settlement by coral larvae, which require solid surfaces to settle,

attach and begin a new colony[37]. In other words, tissue loss diseases can cause rapid mortality and leave the affected area unfavorable for supporting the growth of new coral structures. Tissue loss disease-induced reef degradation can be exemplified in the Caribbean, a 'hot spot' for coral disease, where coral cover has dropped from ~50% in 1970 to 10% in 2002 in large part due to widespread *Acropora* White Pox disease outbreaks[38,39]. Additionally, spikes in other tissue loss diseases such as Black Band, White Pox, White Band, White Plague and Yellow Band have been observed[40]. In the Florida Keys National Marine Sanctuary, reefs have experienced average declines in *Acropora palmata* coverage of 87%[41]. Caribbean reefs are less diverse than most Indo-Pacific reefs. Only three species of *Acropora* can be found in the Caribbean today, while over 300 exist in the Indo-Pacific[1,42]. The extensive losses of coral cover in areas like the Caribbean and the Florida Keys stand as somber examples of the consequences if tissue loss diseases are left unaddressed and understudied.

Coral disease in the Indo-Pacific and Hawaii

The worldwide spread of coral disease has daunting implications in the Indo-Pacific, where many island nations rely on tourism, often largely reef based, to support economies[43]. In Hawaii, tourism accounts for nearly 21% of the total economy[44,43]. In countries such as Palau and the Cook Islands tourism makes up 50% or more of the total GDP[45]. In addition to the economic boost coral reefs afford, reefs harbor some of the highest levels of primary production in the ocean. Coral reefs are responsible for fixing 0.4-5.5 kg of carbon per square meter per year, on the same order as the total above ground primary production of 34 grasslands, 14 tropical forests, and 5 boreal forest sites distributed worldwide[46]. Since the Indo-Pacific houses the majority of corals worldwide, loss of coral cover could lead to drastic reductions in oceanic

primary production, and ultimately increased atmospheric CO_2. As the challenge of climate change continues to mount, Indo-Pacific corals have the potential to help mitigate the problem, if they can be protected.

Baseline levels of coral disease throughout the Indo-Pacific have been low relative to the Caribbean. This is likely due to the increased diversity found on Indo-Pacific reefs. Many tissue loss diseases, especially those caused by infectious agents, are host specifics[5]. Thus, in areas of low diversity like the Caribbean, a greater percentage of the total coral cover would be susceptible if a disease outbreak were to occur. Initially, reports of coral disease from the Indo-Pacific have come primarily from the Great Barrier Reef, where disease was first observed in 1973[47]. In recent years, instances of other tissue loss diseases have begun to appear. To avoid the confusion of subjective evaluation of disease diagnosis, Indo-Pacific coral disease researchers have called all progressive tissue loss diseases of unknown etiology 'White Syndrome' due to the exposure of the white skeletal tissue. A study from 1998-2003 that monitored White Syndrome occurrence across the Great Barrier Reef reported a 20-fold increase over the surveillance period[48]. White syndromes are not limited to the Great Barrier Reef, as reports have been published from American Samoa[49], Palau[50], Marshall Islands[50], Line Islands[51,52], and the Hawaiian Islands[52,53].

Coral disease baselines in Hawaii have mimicked the trends seen elsewhere in the Indo-Pacific, but since 2002 outbreaks have become more frequent[55,56,57]. The Hawaiian Islands, stretching from Kure Atoll (the northwestern most Hawaiian island) to the island of Hawaii (the southeastern most island and part of the main Hawaiian islands) are unique in that they encompass reefs with varying biological, climatic and anthropogenic conditions[58]. The Northwestern Hawaiian Islands (NWHI) (from Nihoa to Kure Atoll) are uninhabited and do not

face the same stresses, such as overfishing, coastal development and sewage spills, as reefs that exist near the large human populations found within the main Hawaiian Islands (MHI). Differences exist between coral diversity in the MHI and NWHI. Specifically, *Acropora* species can be readily found in the Northwestern Hawaiian Islands but are rare in the main islands[54,55,57]. Disease distribution and prevalence also display a geographical difference. *Porites* trematodiasis is more prevalent in the NWHI whereas *Porites* growth anomalies are more prevalent in the MHI[21]. Currently, the tissue loss diseases *Montipora* black band, *Montipora* multi-focal tissue loss, *Porites* multi-focal tissue loss, *Pocillopora* white band, and *Montipora* white syndrome can be found across the Hawaiian archipelago[59].

Montipora White Syndrome

In Hawaii, one disease of concern is *Montipora* White Syndrome (MWS), a progressive tissue loss disease affecting the reef coral *Montipora capitata* (figure 1.1)[59]. While MWS has been reported across the Hawaiian archipelago, it is particularly prevalent in Kaneohe Bay, Oahu[60]. MWS can manifest in two forms: 1) Chronic MWS, a slow progressing form and 2) the faster acting Acute MWS[61]. Chronic Montipora White Syndrome (cMWS) is defined as a chronic progressive infection that causes mortality at a rate of approximately 3% per coral colony per month[60]. Acute Montipora White Syndrome (aMWS) is also a progressive infection but has a comparatively higher and faster rate of mortality than cMWS. The first outbreak of MWS was seen in Kaneohe Bay in 2006, and, since then, numerous outbreaks have occurred[23]. Two significant outbreaks of aMWS occurred in 2010 and 2012, in which 388 and >1000 *M. capitata* colonies were affected, respectively[23]. Research has identified three bacterial pathogens capable of inducing MWS in a lab setting. The first identified agent of MWS was *Vibrio owensii*

strain OCN002, which was shown to induce cMWS approximately 28 days post inoculation in 53% of corals tested[24]. *Pseudoalteramonas* sp. strain OCN003 has been demonstrated to induce aMWS at a rate ~23% of inoculated corals after an average of 21 days[62]. Interestingly, OCN003 has been recently shown to rapidly (<4 days) induce aMWS in corals displaying signs of cMWS[62]. *Vibrio coralliilyticus* strain OCN008 represents the third identified causative agent of MWS[61].

Figure 1.1. Example of Montipora white syndrome (MWS). Healthy *Montipora capitata* in the field surrounds the white skeleton of an MWS affected colony (black arrow). A neighboring colony of *Porites compressa* is in direct contact with the affected *M. capitata* colony yet shows no signs of tissue loss (white arrow). Scale bar represents 10 cm.

Coral Pathogens and mechanisms of virulence

Despite the large increases in coral disease outbreaks worldwide, only a handful of biotic coral pathogens have been identified[5]. Identified pathogens range from bacterial pathogens[24, 52, 61,63–67], to eukaryotic pathogens such as algae, fungi and larval nematodes[68–75, 76–78]. Bacterial pathogens identified and shown to have fulfilled Koch's postulate include *Aurantimonas coralicia*[66], *Serratia marcescens*[38] *Vibrio shiloi*[27], *Vibrio owensii*[24], and *Vibrio coralliilyticus*[29, 52,61]. *A. coralicida* was isolated from a colony of *Dichocoenia stokesi* and was shown to cause White Plague type II in some Caribbean corals[66]. *S. marcescens* was identified as a predominant cause of White Pox in *Acropora palmata* colonies throughout the Florida Keys and Caribbean. It was discovered that sewage systems in the area were inadequate, allowing wastewater containing *S. marcescens* to leak into the marine environment[38]. *V. shiloi* was one of the earliest identified coral pathogen, shown to cause bleaching in the coral *Oculina patagonica*[27,28]. As mentioned above, *V. owensii* has been shown to fulfill Koch's postulates with cMWS in Kaneohe Bay[24]. Three strains of *V. coralliilyticus* have been demonstrated to fulfill Koch's postulates. Strain BAA-450 can cause bleaching or tissue lysis in *Pocillopra damicornis* in a temperature-dependent fashion[29], strain OCN014 was shown to induce *Acropora* white syndrome in *Acropora cytherea* in Palmyra Atoll[52], and OCN008 is a causative agent of aMWS in *Montipora capitata* in Kaneohe Bay, Oahu[61]. While in many cases Koch's postulates cannot be satisfied due to disease causing consortia or uncultivable pathogens, the number of bacteria definitively shown to be pathogens is surprisingly low, and the number of demonstrated virulence factors is even scarcer. This is likely due to the lack of research in this field rather than a limited number of true pathogens.

Of the above pathogens, only four virulence factors have been reported: Toxin P production, flagellar motility, and the genes *toxR* and *mshA*. *V. shiloi* is the only coral pathogen in which an infection model has been established[28]. First, the bacterium senses and moves towards the coral *O. patagonica* via chemotaxis, or is delivered to the coral via a marine fireworm. After bacterial replication, Toxin P is produced by the *V. shiloi* community which leads to the loss of *Symbiodinium* and therefore bleaching[27,28]. Production of flagella was shown to be required for the infection of *P. damicornis* by a strain of *V. coralliilyticus* closely related to BAA-450[29]. Lastly, recent work in *V. coralliilyticus* OCN014 has identified genes homologouss to *mshA* and *toxR* of *Vibrio cholerae* as virulence factors[52]. To date no other virulence factors have been published in the field of coral disease, again highlighting the scarcity of research and the need to better understand how corals become infected. Only then can we begin to start constructing plans to mitigate these diseases.

Ushijima et. al[61] isolated *Vibrio coralliilyticus* strain OCN008 and demonstrated it capable of inducing aMWS in the lab. Infection occurred between 12 hours and 4 days post inoculation, with mortality occurring in 80-90% of inoculated colonies. OCN008 was initially isolated from, healthy *Porites* coral and it was noted that colonies that appeared to inhibit the growth of neighboring bacteria. The strains isolation from healthy coral may indicate its constant presence in the environment, or that it is part of the constituent holobiont of certain Kaneohe Bay corals. After isolation and identification, it was confirmed that OCN008 induced signs of MWS in the lab, and produced a broad spectrum antibiotic. This was of particular interest since (1) OCN008 is one of only a handful of identified bacteria demonstrated to infect coral and (2) OCN008 is the only coral pathogen identified that produces an antibiotic. Further

examination revealed a cell density dependent nature of antimicrobial production leading to the investigation into quorum sensing in this pathogen.

Quorum Sensing in the Genus Vibrio

The quorum sensing networks in *Vibrio harveyi* and *Vibrio cholerae* have been well characterized and are shown in figure 1.2[79–81]. Briefly, QS in these organisms involves parallel pathways, each consisting of an autoinducer (AI) synthase and an inner-membrane-bound histidine kinase receptor. Each pathway leads to the activation of one of the two main QS regulators: AphA, which is active at low cell density (LCD), and LuxR/HapR, which is active at high cell density (HCD)[82]. In *Vibrio harveyi*, the synthase and receptor genes are: *luxM* which synthesizes AI-1, an acylated homoserine lactone (AHL) that binds *luxN*[83]; *luxS* which synthesizes the precursor to AI-2[84], a furanosyl borate diester sensed by *luxPQ* in *V. harveyi*; and *cqsA* which synthesizes the autoinducer CAI-1, which is sensed by *cqsS*[85]. *Vibrio cholera* does not possess the AHL pathway but does possess another distinct pathway known as the *varA* / *varS* system, although the synthase and AI molecule of this pathway have yet to be identified[86].

At low cell density, when few AI molecules are bound to their corresponding sensors, the histidine kinase receptors auto-phosphorylate, which results in the phosphorylation of LuxO via the phospho-relay protein LuxU[87]. The phosphorylated form of LuxO (LuxO~P), in conjunction with σ^{54} promotes the transcription of a number of small RNAs known as the Qrrs (Quorum Regulating RNAs)[88]. The Qrrs, with the help of the RNA chaperone Hfq, inhibit the transcription and translation of the HCD master regulator of QS, LuxR (HapR in *Vibrio cholerae*) and promote the transcription and translation of LCD master regulator AphA[88]. It should be noted that Qrr RNAs function by directly base-pairing to their RNA / DNA targets,

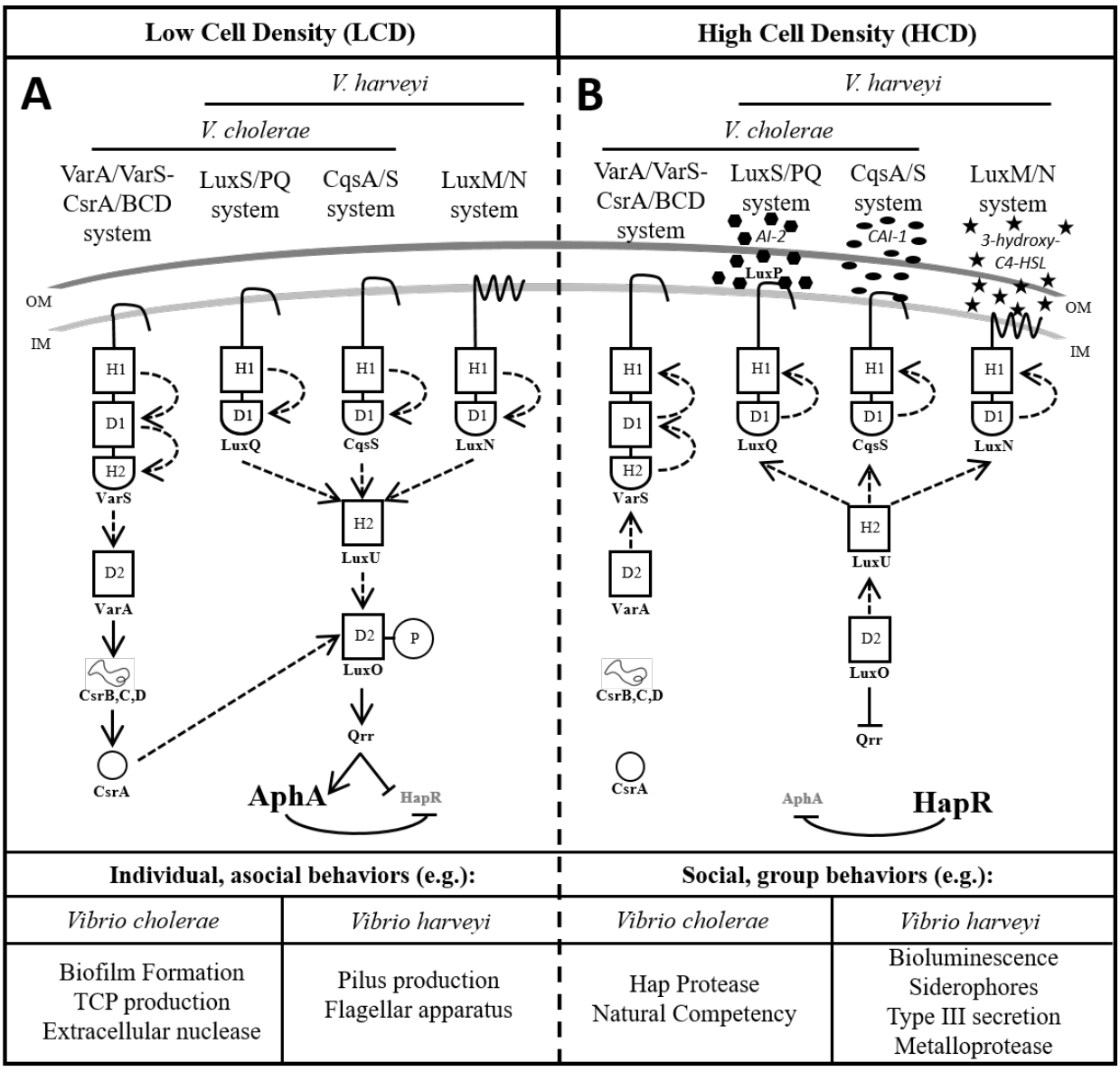

Figure 1.2. Quorum Sensing in Vibrio cholerae and Vibrio harveyi. Each bacterium employs three QS systems, two of which are shared between the two species. Horizontal lines above the system indicate which pathways exist in each organism. Dashed lines indicate the flow of phosphorylation. **A)** Low Cell Density (LCD). At LCD, extracellular autoinducer (hexagons, ovals, and stars) concentration is low and histidine kinase receptors are unbound. This induces a phospho-relay pathway ending with a phosphorylated, transcriptionally active LuxO. The phosphorylated LuxO then in-directly induces expression of the LCD master regulator AphA and repression of the HCD master regulator HapR. **B)** High Cell Density (HCD). At HCD, extracellular autoinducer concentration is significant enough to bind their cognate histidine kinase receptors, inducing a reverse in the phospho-flow. This results in a de-phosphorylated LuxO that becomes transcriptionally inactive. AphA expression is then repressed and HapR becomes the dominant regulator of the system. Some classical QS behaviors for each organism at each state are listed below the diagram.

and that binding regions of the Qrr RNAs are highly conserved across *Vibrio* species[89]. Five Qrrs have been identified in *Vibrio harveyi* (Qrrs1-5) and four in *Vibrio cholerae* (Qrrs1-4)[89]. At high cell density, the sensor kinases are saturated with AIs, which causes the sensors to abort kinase activity and begin phosphatase activity, resulting in the de-phosphorylation of LuxU, and ultimately LuxO. The un-phosphorylated form of LuxO is inactive and cannot promote the transcription of the Qrr RNAs. This results in an increase in the levels of LuxR/HapR and a decline in the levels of AphA, resulting in large shift in gene expression. LuxR/HapR and AphA control large regulons and have been shown to act as both activators and inhibitors. In *Vibrio harveyi*, 625 genes are controlled by LuxR, 167 genes are regulated by AphA, and 77 genes are co-regulated by both LuxR and AphA[90]. In both *Vibrio harveyi* and *Vibrio cholerae*, AphA and LuxR/HapR show inverse profiles in relation to cell density: AphA is present and active at low cell density, and LuxR/HapR is present and active at high cell density.

It should be noted that the VarA/VarS pathway of *Vibrio cholerae* is distinct from the other aforementioned pathways. At LCD the histidine kinase receptor, VarS, activates the response regulator VarA via phosphorylation, which activates the transcription of three small RNAs named CsrBCD[86,91]. These sRNAs are distinct from the Qrr RNAs also present in the canonical regulatory network. The presence of the CsrRNAs prevent the activation of the global regulatory protein CsrA, which, when active, inhibits LuxO~P (the active form of LuxO). Thus, at HCD, CsrBCD are not transcribed, CsrA is active, and LuxO is incapable of promoting the Qrrs, resulting in an increase in HapR and a switch to HCD behavior. So, the VarA / VarS system controls QS through LuxO independently of LuxU and the other defined QS pathways of *Vibrio cholerae* (LuxPQ/S and CqsA/S).

Current Work

This dissertation includes work that investigates the quorum sensing pathways of *Vibrio coralliilyticus* strain OCN008 and its role in pathogenesis. First, a classical genetic approach was taken to find genes involved in the regulation of andrimid production. Second, a forward genetic approach was used to search for other genes involved in quorum sensing involved in andrimid production and pathogenesis based on homologies to defined quorum sensing networks. Lastly, mechanics of quorum sensing were investigated via protein analysis of the master regulator of quorum sensing in *Vibrios.*

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CHAPTER TWO: ANDRIMID PRODUCTION IS REGULATED BY THE AI-2 MEDIATED LUXPQ/S QUORUM-SENSING CIRCUIT IN THE HAWAIIAN CORAL PATHOGEN *VIBRIO CORALLIILYTICUS* STRAIN OCN008

INTRODUCTION

Coral disease represents a serious threat to the economies and wellbeing of coastal and island populations worldwide. While coral disease encompasses many types of conditions, tissue loss diseases have the potential to devastate coral reef ecosystems at frightening rates. The effect of coral disease is well demonstrated by the fate of acroporid corals in the Caribbean, where multiple outbreaks of the tissue loss diseases *Acropora* White Pox and *Acropora* White Plague (types I and II) have occurred since the mid 1970s[6, 12, 38, 40, 65,92,93]. In many cases of disease outbreaks, the losses have been extreme. One study examined survey data of total *A. palmata* and *A. cervicornis* coverage (m^2) at Looe Key, Florida, between 1983 and 2000, and found that coverage had declined by 93% and 98%, respectively, over the study period[41]. These large-scale coral disease outbreaks brought on by infectious pathogens often occur following periods of abnormal environmental stresses[94–96]. Increasing frequency of climate change events that induce abiotic stresses threatens to bring about similar damage in areas that, so far have not seen such drastic declines in coral cover. This highlights the need to study and understand the causative agents and mechanisms behind coral disease if there is any chance to prevent similar losses.

Historically, coral disease prevalence in the Indo-Pacific and Hawaii has been low relative to the Caribbean[97]. Over the past decade, however, incidents of White Syndrome

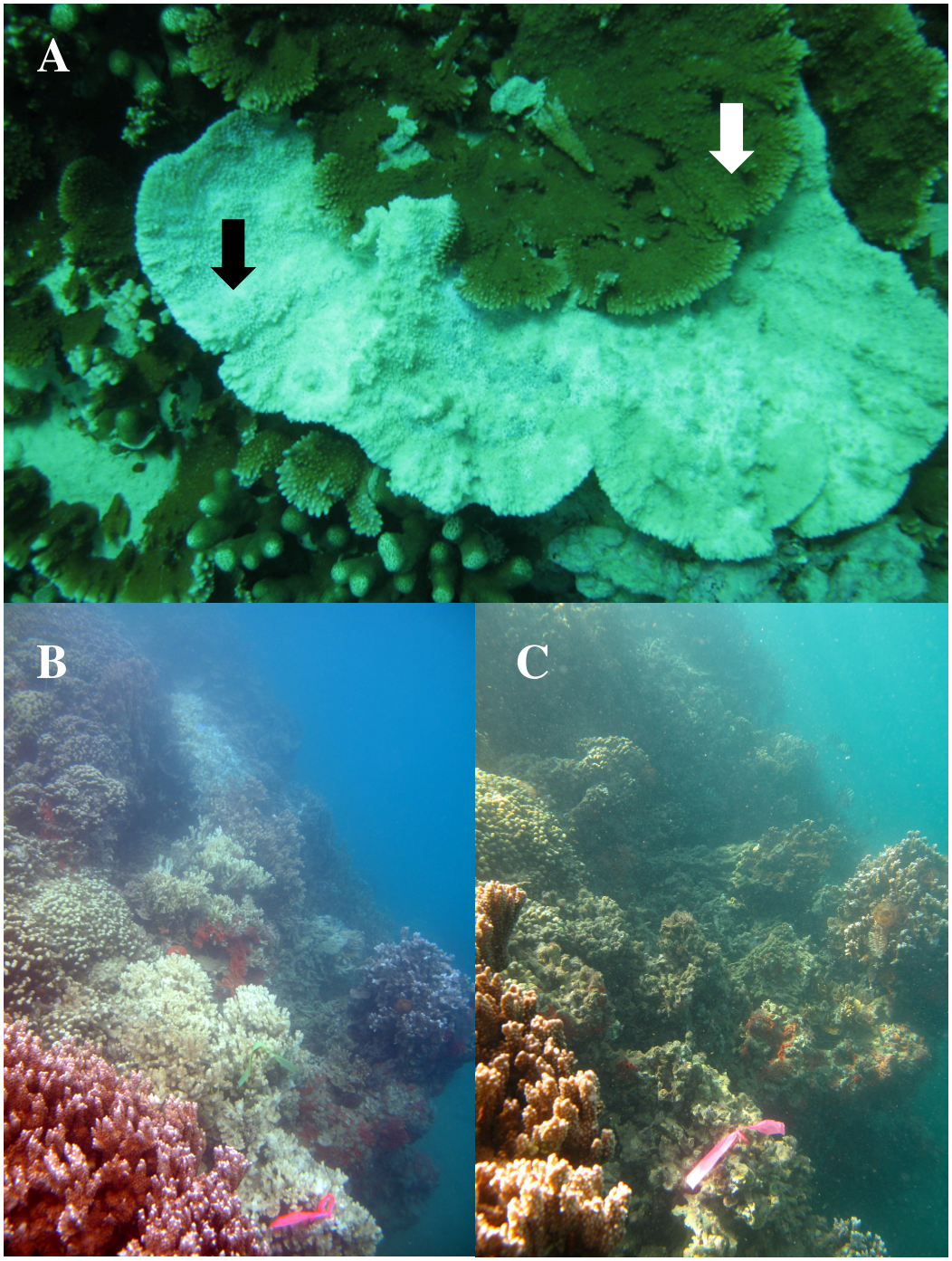

Figure 2.1. Acute Montipora White Syndrome. A) Close up of a *Montipora capitata* colony affected with aMWS. Bare skeleton is exposed (black arrow) surrounded by healthy tissue (white arrow). **B)** Reef slope affected by aMWS near Lilipuna pier in Kaneohe Bay, Oahu. This picture was taken in April 2010. Note the appearance of disease transmission, as the infection seems to have spread across the reef. **C)** The same reef slope (note the pink tag) one year later (April 2011). All aMWS affected corals have been reduced to rubble.

outbreaks (a general term for progressive tissue loss diseases of unknown etiology in the Indo-Pacific) have become more common[54, 56,59]. In Kaneohe Bay, Oahu, the reef coral *Montipora capitata* has been particularly affected by White Syndrome, with outbreaks of two distinct forms of *Montipora* White Syndrome (MWS) observed within the last 10 years[23]. The most recent outbreaks of MWS have been called Acute MWS (aMWS) due to rapid progression of the disease legion (figure 2.1A). This form of MWS has the potential to cause significant damage to the reef, as demonstrated by photos of a reef slope adjacent to Lilipuna Pier in Kaneohe Bay during an outbreak event and the same location one year later (figure 2.1B). The disease was species-specific and progressed in a linear fashion, consistent with an infectious disease. The result of the pictured outbreak was complete loss of coral cover in many affected areas leaving behind silt and rubble. This result, in addition to the loss of the ages old coral, also yields the area unsuitable for new coral growth, as coral larvae require solid surfaces on which to settle and attach[98]. Clearly this disease possesses the capacity to cause massive, wide scale coral mortality within Hawaii and there is a dire need to study this disease process to prevent the massive coral reef loss that has occurred across the Caribbean.

Recently, *Vibrio coralliilyticus* strain OCN008 was identified as an etiological agent of aMWS in Kaneohe Bay[61]. Interestingly, OCN008 produces the antibiotic andrimid as a virulence factor[99]. OCN008 is not readily found in the water column, but OCN008-like isolates have been found on some reef fish and invertebrates[100]. Additionally, the original isolate of OCN008 came from a healthy fragment of the reef coral *Porites compressa* from Kaneohe Bay. Thus, OCN008 can be found in the reef environment but likely not at high enough levels to cause infection without other contributing factors. It has been hypothesized that infection begins with a shift in the normal micro flora found on the coral surface[101]. Coral surface associated

microorganisms are the corals' first line of defense and can prevent colonization by harmful bacteria via competitive exclusion[102,103]. If a stress event occurs, such as fresh water input (a large rain event) or a period of increased sea surface temperatures, this protective community can be compromised, creating an opening for pathogens like OCN008 to attach to the coral[104]. If OCN008 is able to initially colonize a coral colony, it is believed that andrimid is subsequently produced, which prevents the proliferation of nearby coral bacteria. This creates even more uninhabited space for OCN008 to fill and, ultimately, overtake host defenses resulting in acute tissue loss disease.

Bacterial members of the genus *Vibrio* are well known as disease causing agents. Infectious species include the human pathogens *V. cholerae, V. parahemolyticus* and *V. vulnificus*, the fish pathogens *V. anguillarum* and *V. salmonicida,* and invertebrate pathogens *V. harveyii, V. splendidus* and *V. coralliilyticus*[29,105–116]. *Vibrios* are known, aside from their pathogenic tendencies, for quorum sensing (QS) – cell density regulated population behavior. The first description of QS was in *V. fisheri* (a symbiont of the Hawaiian Bobtail squid), and was found to regulate the induction of bioluminescence in a cell density dependent manner[117]. Since then, QS systems have been found to be nearly ubiquitous amongst *Vibrios,* and has been found to occur in numerous other genera and even phyla of bacteria. Using QS, *Vibrios* are able to execute large shifts in expression profiles in response to local cell density. Put most simply, populations exhibit changes from low cell density (LCD) to high cell density (HCD) behavior. The QS circuits of *V. harveyi* and *V. cholerae* are shown in figure 2.2.

QS is thought to have evolved as it provided benefits to bacteria when acting as groups that could not be achieved by individuals acting alone. Importantly, QS has been repeatedly associated with virulence in human pathogenic *Vibrios*[118]. It has been hypothesized that, in

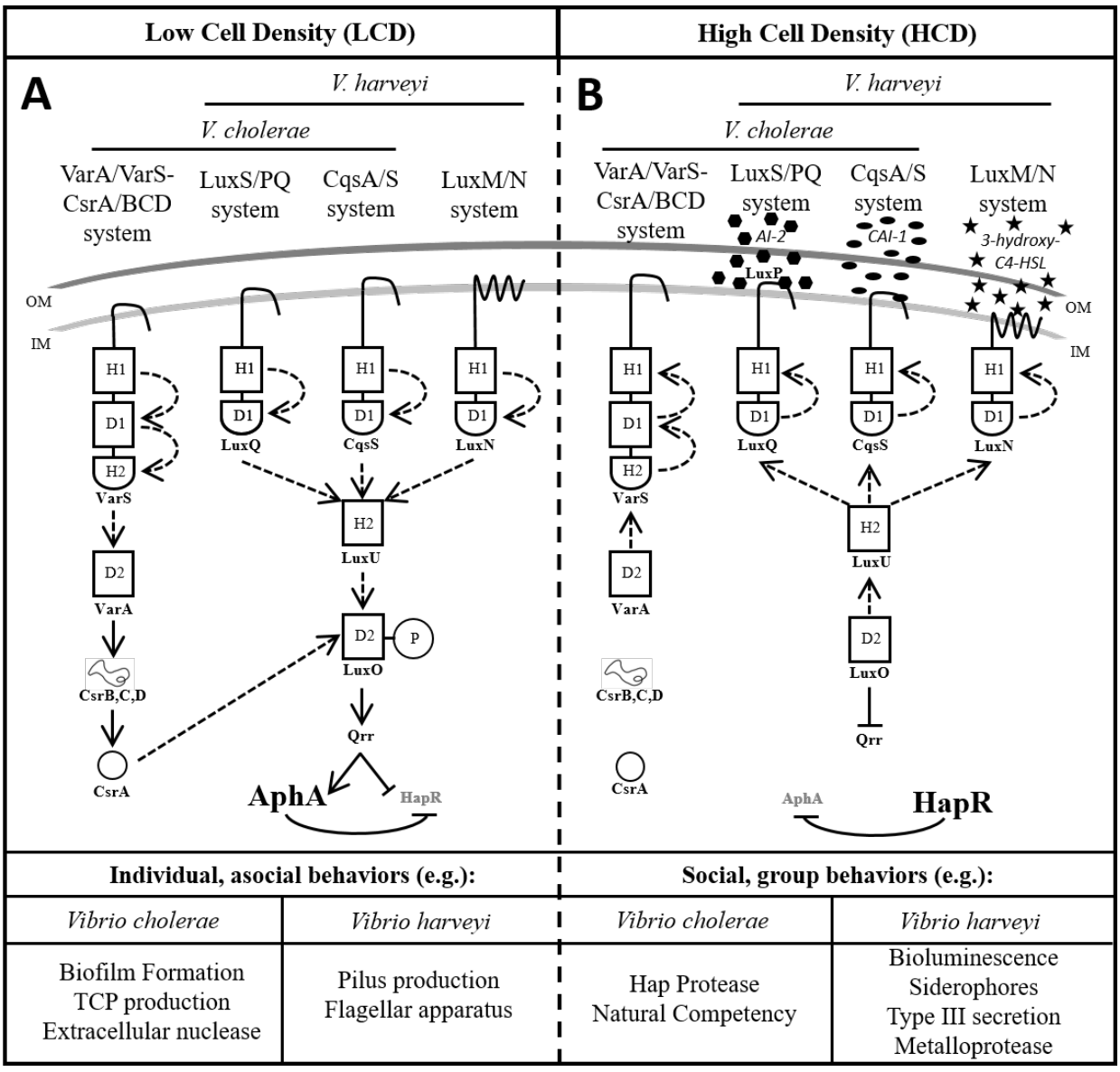

Figure 2.2. Quorum Sensing in Vibrio cholerae and Vibrio harveyi. Each bacterium employs three QS systems, two of which are shared between the two species. Horizontal lines above the system indicate which pathways exist in each organism. Dashed lines indicate the flow of phosphorylation. **A)** Low Cell Density (LCD). At LCD, extracellular autoinducer (hexagons, ovals, and stars) concentration is low and histidine kinase receptors are unbound. This induces a phospho-relay pathway ending with a phosphorylated, transcriptionally active LuxO. The phosphorylated LuxO then in-directly induces expression of the LCD master regulator AphA and repression of the HCD master regulator HapR. **B)** High Cell Density (HCD). At HCD, extracellular autoinducer concentration is significant enough to bind their cognate histidine kinase receptors, inducing a reverse in the phospho-flow. This results in a de-phosphorylated LuxO that becomes transcriptionally inactive. AphA expression is then repressed and HapR becomes the dominant regulator of the system. Some classical QS behaviors for each organism at each state are listed below the diagram.

many cases, the production of certain virulence factors is only effective when the density of cells is sufficient to induce the required ratio of virulence factor proteins to host cells needed to affect the host organism. For example, production and secretion of an antibiotic is only beneficial if the extracellular concentration of the antibiotic will exceed the minimal inhibitory concentration (MIC) of the surrounding organisms. In many cases, studying the QS circuits of pathogenic strains has led to the discovery of mechanisms of pathogenesis. This has made QS a target for many disease prevention strategies. For example, potato soft rot and tomato crown gall diseases (significant to the agricultural industry) were successfully prevented via the biologically induced degradation of pathogen specific QS molecules[119,120].

Coral disease is a vastly understudied field, with only a handful of pathogens and even fewer virulence factors identified. Since OCN008 is a member of the genus *Vibrio,* QS is presumably employed to some degree in its ability to cause coral disease. The availability of described QS circuits in other *Vibios* provides a great starting point to begin to dissect the mechanisms of OCN008 induced aMWS. The objective of this study was to examine the relationship between QS in OCN008 and the production of the novel virulence factor andrimid. As it has been previously shown that andrimid is utilized as virulence factor, understanding its regulation may yield more insights into the infectious process and potentially even lead to preventative strategies.

MATERIALS AND METHODS

Bacterial Growth Conditions

All strains and plasmids used in this study are listed in Table 1. Marine bacteria were grown at 28°C in glycerol artificial sea water media (GASW[121]), with the exception that Instant

Ocean® salt mix (Spectrum Brands, Atlanta, GA.) was used instead of Rila salts. Thiosulfate Citrate Bile Salts Sucrose (TCBS) solid media was made according to manufacturers instructions (BD, Franklin Lakes, NJ.) *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Concentrations of antibiotics were 15 (for *E. coli* strains) or 30 (*Vibrio* strains) μg/mL for chloramphenicol, 15 μg/mL for gentamycin, 50 μg/mL for kanamycin, 100 μg/mL for spectinomycin, and 50 μg/mL for streptomycin. For solid media, 15 g/L of Agar was added prior to autoclaving. Growth media for E. coli auxotrophic strains were supplemented with deoxythymidine (DT) or diaminopimelate (DAP) at a final concentration of 0.3 mM as required. Arabinose-inducible expression of the ccdB gene was achieved by the addition of 0.2% L-arabinose to the growth medium, and expression was repressed by addition of 1% D-glucose. For boron containing media, 0.4% boric acid was added.

Assessment of Andrimid Production

Production of andrimid was assessed based on the ability to inhibit the growth of the target bacteria *Alteramonas* sp. strain OCN004 (solid media assays) or *Pseudoalteramonas* sp. strain OCN003 (liquid media assays). Both strains can be readily cultured on GASW medium from healthy coral. For solid media assays, GASW plates were spread with 50 μL of OCN004 that had been grown overnight. The strain to be assayed was grown up overnight, diluted 1:1000, and 10 μL was spotted onto the plate. Solid media assays were mostly qualitative, scored as positive or negative based on the appearance of a zone of inhibition after 24 hours of growth. For liquid assays, the assayed strains were grown for 24 hours with aeration. After growth, cells were removed via filtration with 0.2 μm filter. This cell free conditioned media was then serially diluted and inoculated with one μL OCN003 that had been grown over night.

After 24 hours the optical density of each dilution was read at 600 nm (OD_{600}) using a spectrophotometer. For the andrimid production over the growth curve assay, dilutions and growth were done in 5mL sterile glass tubes. The liquid screen performed on all mutants was done using 96 well plates and a Tecan plate reader.

Transposon Mutagenesis and inverse PCR

The Mariner Transposon containing plasmid pBT20 was conjugated from SM10 λ Pir E. coli to OCN008 overnight on GASW media. Donor and recipient strains were grown overnight, diluted 1:1000, and grown to an OD~1.0. Cells were then washed twice with fresh GASW, combined, and 50 μL was spotted into the center of a GASW plate. Selection for transposon mutants was achieved by plating the re-suspended and twice washed (with GASW) conjugation spots onto TCBS supplemented with 15 μg/mL of gentamycin. Resulting colonies were then patched onto GASW plates containing a lawn of the target bacterium *Alteramonas* sp. strain OCN004. Isolates found not to produce a zone of inhibition were then purified via growth on TCBS, and stored at -80°C in 80% glycerol.

Transposon insertions were mapped using inverse PCR. Briefly, genomic isolation was performed as previously described (reference for genomic prep of marine bacteria). 10 μL of the resulting genomic DNA was digested with the restriction enzyme HpyCH4IV (New England Biolabs). Digestion was stopped by incubation at 80°C for 30 minutes, and T4 DNA Ligase (Roche) was added to induce self ligation. PCR was run using the primers HpyCH4IV-F and HpyCH4IV-R. Sanger sequencing off of successful inverse PCR products was done using the HpyCH4IV-F primer.

Plasmid and Strain Creation

Plasmids and strains used in this study are listed in table 2.1. Primers can be found in table 2. Strains lacking chromosomal genes were constructed using the method described by Le Roux, et. al[122]. Briefly, suicide vectors based on pSW4426T[122] were conjugated into *Vibrio coralliilyticus* via tri-parental conjugations. DT auxotrophic π 3813 E. coli carrying the self transmissible plasmid pRK2013, DAP auxotrophic *E. coli* carrying deletion suicide vectors and OCN008 were grown over night, diluted 1:1000 in fresh media, and grown to an OD_{600} of ~1.0. Cells were then washed three times with fresh GASW, combined, and 50 μ L of the corresponding mixture was spotted onto a GASW plate supplemented with 1% D-glucose and 0.3M DT and DAP. The spots were allowed to conjugate overnight, and the spots were washed twice with fresh GASW and plated for selection of single recombinants on either TCBS or GASW supplemented with 30 μ g/mL chloramphenicol and 1% D-glucose. Resulting colonies were streaked onto GASW 1% D-glucose plates containing spectinomycin and streptomycin. Resulting colonies were grown overnight in liquid GASW 1% D-glucose overnight without selection, and plated onto GASW plates supplemented with 0.2% arabinose to select for double recombinants. The resulting colonies were screened for mutations using primers outside of the mutation locus. Mutations were verified using the same outside primers. In the case of the *luxO(D47E)* mutation the mutation was verified by sequencing the chromosomal locus using the primer luxO(D47E)-seq. Complementation vectors were conjugated into corresponding mutants using the same strategy described above.

Plasmid pPJAV250 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *aphA*. Regions up- and downstream of *aphA* were amplified by PCR from chromosomal DNA with the primer pairs aphA-up-BamHI-F and

aphA-up-SmaI-R and aphA-dn-SmaI-F and aphA-dn-NruI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+ (Stratagene). The up- and downstream regions were cloned as an *Eco*RI-*Spe*I fragment into the *Eco*RI-*Xba*I sites in pSW4426T.

Plasmid pAHB105 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *cqsS*. Regions up- and downstream of *cqsS* were amplified by PCR from chromosomal DNA with the primer pairs cqsS-up-MunI-F and cqsS-up-SmaI-R and cqsS-dn-SmaI-F and cqsS-dn-MunI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The up- and downstream regions were cloned as an *Mun*I fragment into the *Eco*RI site in pSW4426T.

Plasmid pAHB102 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *hapR*. Regions up- and downstream of *hapR* were amplified by PCR from chromosomal DNA with the primer pairs hapR-up-EcoRI-F and hapR-up-SmaI-R and hapR-dn-SmaI-F and hapR-dn-EcoRI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+ (Stratagene). The up- and downstream regions were cloned as an *Eco*RI fragment into the same site in pSW4426T.

Plasmid pPJAV253 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *luxN1*. Regions up- and downstream of *luxN1* were amplified by PCR from chromosomal DNA with the primer pairs luxN1-up-BamHI-F and luxN1-up-SmaI-R and luxN1-dn-SmaI-F and luxN1-dn-SacI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I

site of pBlueScript SK+. The up- and downstream regions were cloned as an *Eco*RI-*Spe*I fragment into the *Eco*RI-*Xba*I sites in pSW4426T.

Plasmid pAHB104 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *luxN2*. Regions up- and downstream of *luxN2* were amplified by PCR from chromosomal DNA with the primer pairs luxN2-up-EcoRI-F and luxN2-up-SmaI-R and luxN2-dn-SmaI-F and luxN2-dn-EcoRI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The up- and downstream regions were cloned as an *EcoR1* fragment into the same site in pSW4426T.

Plasmid pAHB123 is a suicide vector based on pSW4426T used to delete all but the first 30 nucleotides of the coding region of *luxN2* and last 30 nucleotides of the coding region of *luxN1,* genes located adjacent to one another in the genome. Regions upstream of *luxN2* and downstream of *luxN1* were amplified by PCR from chromosomal DNA with the primer pairs luxN2-up-EcoR1-F and luxN2-up-SmaI-R and luxN1-dn-SmaI-F and luxN1-dn-SacI-R, respectively. The upstream region of *luxN2* and the downstream region of *luxN1* were cloned into the *Eco*RV and *Eco*RV-*Sma*I sites of pBlueScript SK+, respectively, and screened for directionality using PCR. The *luxN2* upstream fragment was then cloned as an *Eco*RV-*Sma*I fragment into pBlueScript SK+ containing the correctly oriented *luxN1* downstream region and checked for directionality using PCR. The entire construct containing the up- and downstream regions of *luxN2* and *luxN1* were cloned as an *Eco*R1-*Cla*I fragment into the same sites in pSW4426T.

Plasmid pPJAV254 is a suicide vector based on pSW4426 used to delete all but the first 30 and last 30 nucleotides of the coding region of *luxP*. Regions up- and downstream of *luxP*

were amplified by PCR from chromosomal DNA with the primer pairs luxP-up-BamHI-F and luxP-up-SmaI-R and luxP-dn-SmaI-F and luxP-dn-SacI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The up- and downstream regions were cloned as an *Eco*RI-*Spe*I fragment into the *Eco*RI-*Xba*I sites in pSW4426T.

Plasmid pPJAV255 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *luxT*. Regions up- and downstream of *luxT* were amplified by PCR from chromosomal DNA with the primer pairs luxT-up-BglII-F and luxT-up-SmaI-R and luxT-dn-SmaI-F and luxT-dn-SacI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The up- and downstream regions were cloned as an *Eco*RI-*Spe*I fragment into the *Eco*RI-*Xba*I sites in pSW4426T.

pAHB124 is a suicide vector based on pSW4426T used to delete all but the first 30 and last 30 nucleotides of the coding region of *luxT*. Regions up- and downstream of *luxT* were amplified by PCR from chromosomal DNA with the primer pairs VarA-up-MunI-F and VarA-up-SmaI-R and VarA-dn-SmaI-F and VarA-dn-MunI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The up- and downstream regions were cloned as a *MunI* fragment into the *Eco*RI site on pSW4426T.

pAHB183 is a suicide vector used make the *luxO(D47E)* mutation. The entire luxO coding region was amplified using the primers luxO-up-MunI-F and luxO-dn-MunI-R and cloned in the *SmaI* site on pBlueScript Sk+. Point mutation was achieved using quick change

PCR[123] with the primers luxO(D47E)-F and luxO(D47E)-R. The mutagenized construct was then cloned as a *MunI* fragment into the *EcoRI* site of pSW4426T.

The complementation vectors pAHB180 and pAHB181 are based on pBU115, a derivative of pEVS78 with a uniquie *SmaI* site added to the MCS. The *luxP* and *luxT* regions were amplified from chromosomal DNA with the primers luxT-up-BglII-F / luxT-dn-SacI-R and luxP-up-BamHI-F / luxP-dn-SacI-R, respectively. The resulting constructs were cloned as PCR products into the *SmaI* site of pBU115.

The *hapR* complementation vector pAHB190 is arabinose inducible and based on pAHB189. pAHB189 is a derivative of pBU115 with the P_{BAD}-*araC* construct from pSW4426T, amplified with the primers araC-ccdB-F and P_{BAD}-araC-NdeI-R, cloned into the *SmaI* site of pBU115 with an added unique *NdeI* site downstream of P^{BAD}. The coding region of *hapR* was amplified using the primers HapR-NdeI-F and HapR-SalI-R and cloned as a *NdeI-SalI* fragment into the *NdeI* and *XhoI* sites on pAHB189.

RESULTS

Andrimid production is cell-density dependent

After initial isolation and identification of OCN008, antimicrobial production was shown to be undetectable at low cell densities, and readily detectable at high cell densities – a behavior consistent with quorum sensing. When andrimid production was screened at two-hour intervals along a 30-hour growth curve, it was undetectable until after 12 hours of growth and remained detectable for the remainder of the experiment. Andrimid production became measurable once OCN008 was in late-log phase and reached on OD of ~2.8. Andrimid production was assessed

based on the ability of conditioned, cell-free liquid media to inhibit the growth of the target bacterium *Pseudoalteramonas* sp. strain OCN003 (figure 2.3).

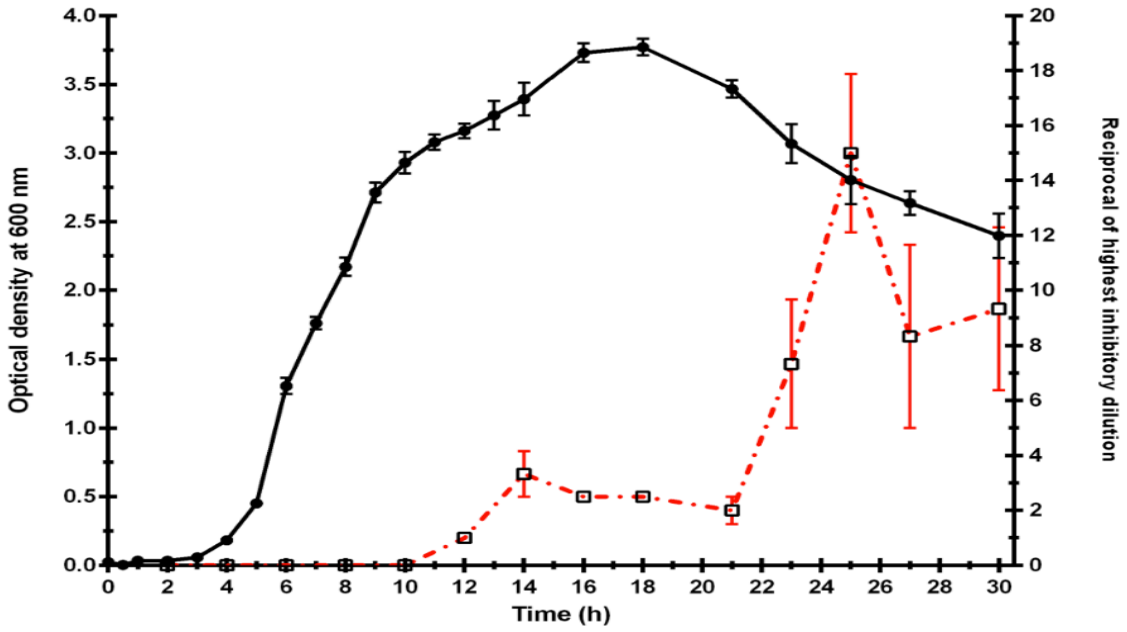

Figure 2.3. Andrimid production is associated with high cell density. The black line represents wild type OCN008 cell density measured by the optical density at 600 nm and the red line represents andrimid production. Andrimid production is shown as the reciprocal of the highest dilution of cell free conditioned media capable of inhibiting growth of the target strain to an OD600 of under 0.01.

Quorum sensing is known to control low and high cell density behaviors in *Vibrios*[80]. In most *Vibrios,* QS is characterized by the shift from LCD to HCD behavior, the observed low/high expression profile is the result of the specific levels of one, or both, regulators. *Vibrios* and other pathogens have been demonstrated repeatedly to regulate the expression of virulence factors at HCD through QS[124–126]. Additionally, numerous organisms use QS to regulate the production of a number of different antibiotics, although this has not been described in any

Vibrio species[127–129]. Thus, the observation that andrimid production appears to be regulated by cell density is consistent with a QS regulated phenotype.

Potential QS genes that regulate andrimid production identified by transposon mutagenesis

V. coralliilyticus strain OCN008 was initially isolated based on the production of a putative antimicrobial compound that inhibited the growth of nearby bacteria on a solid medium. To identify genes necessary for lateral growth inhibition, a transposon screen was used to generate mutants of strain OCN008 that lacked antimicrobial activity. Five of the resulting 11,195 mutants contained transposon insertions in genes homologous to the known quorum sensing genes *aphA, hapR, luxN, luxP,* and *luxT* of *Vibrio cholerae* and/or *Vibrio harveyii*. The gene designations in strain OCN008 were ERB64015, ERB64458, ERB62642, ERB63170, and ERB66350, respectively. AphA and HapR are the low and high cell density QS master regulators, respectively. LuxN and LuxP (in conjunction with LuxQ) are histidine kinase receptors that respond the QS auto-inducers AI-1 and AI-2, respectively. LuxT is annotated as a transcriptional regulator, with little published information regarding its role in QS, despite being repeatedly implicated in the process [130–133]. Colonies of each of the five transposon mutants failed to create a zone of inhibition of growth of the target bacterium A*lteromonas* sp. strain OCN004 (a strain of bacteria easily cultured from *M. capitata* coral mucus). In contrast, the wild-type OCN008 prevented growth within approximately 4 mm of colonies on solid medium.

Transposons create random, marked mutations throughout a genome via the insertion of a DNA element into the chromosome. While incredibly useful for screening large numbers of mutants for testable phenotypes (*i.e*. antimicrobial production), the nature of the mutation can affect the expression of genes neighboring the insertion in addition to the gene that was mutated.

This is known as a polar effect. In this case, the gene identified via transposition would falsely appear to be involved in the process under investigation. To verify that the observed andrimid null phenotype of the transposon mutants was the result of the disruption of the above genes, rather than a polar effect, clean, in-frame deletion mutants were created for each gene via allelic replacement[134]. Clean deletions were designed such that the first and last 10 amino acids were left unaltered, and were chosen in an attempt to minimize the chance of disrupting the expression of non-targeted genes.

Andrimid production was assessed based on growth inhibition of A*lteromonas* sp. strain OCN004, hereafter referred to as the target strain, on solid media. OCN008 and the QS mutants were spotted onto plates containing a lawn of the target bacterium and assessed for the presence of zones of inhibition after incubation. The Δ*hapR,* Δ*luxP* and Δ*luxT* mutants displayed abrogated andrimid production while the Δ*aphA* and Δ*luxN* mutants inhibited the growth of the target strain in a manner comparable to the wild type (figure 2.4). To further verify that the deleted coding regions were responsible for the andrimid null phenotypes, the Δ*hapR,* Δ*luxP* and Δ*luxT* strains were each genetically complemented with plasmid born, full-length copies of the deleted genes. In each case, complementation restored the production of a zone of growth inhibition similar to that produced by the wild type. In summary, the LuxPQ/S system, the high cell density master regulator HapR and the QS transcriptional regulator LuxT are required for andrimid production. The LuxM/N AHL pathway does not appear to be involved.

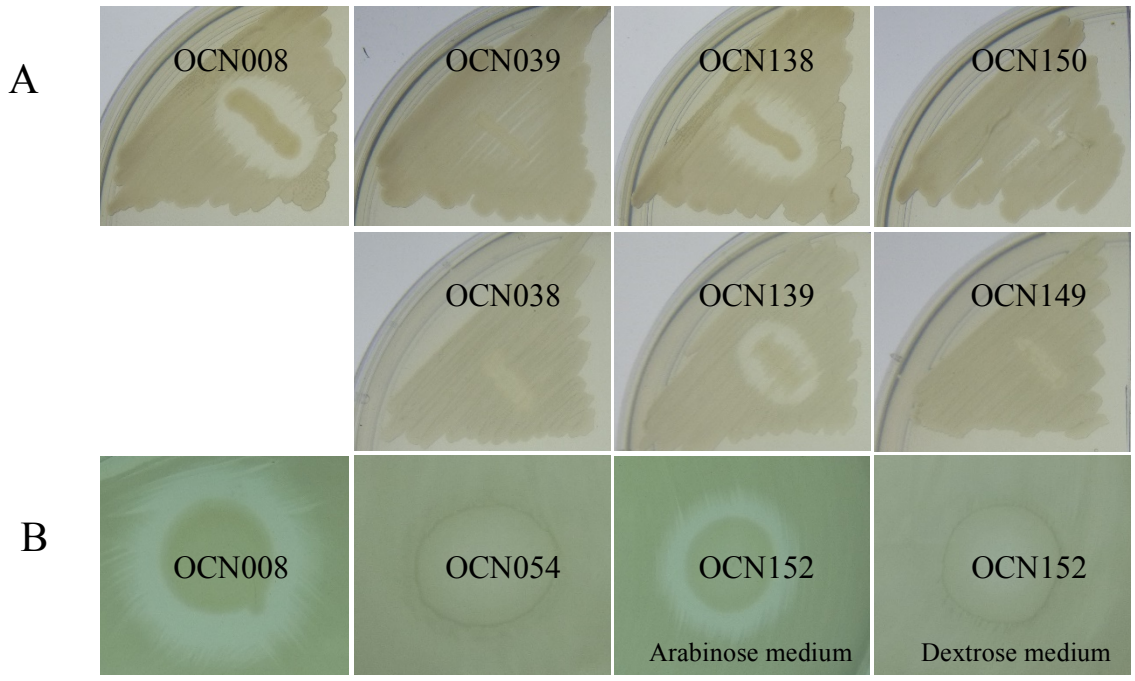

Figure 2.4. Andrimid inhibition of a target bacterium by wild type OCN008 and QS deletion mutants from sub-aim one. **A)** Inhibition by wild type OCN008, OCN039 (Δ *luxT)*, OCN138 (complemented Δ l*uxT*), OCN150 (OCN039 + pBU115 the empty complementation vector negative control), OCN038 (Δ *luxP*), OCN139 (complemented Δ *luxP*) and OCN149 (OCN038 + pBU115). **B)** Inhibition of the target bacterium by OCN008, OCN054 (Δ *hapR*) and OCN152 (OCN054 complemented with the HapR CDS under control of the arabinose promoter P_{BAD}) on media containing either arabinose or glucose.

Reverse genetics revealed other possible QS genes in the genome of OCN008.

The quorum sensing pathways of *Vibrio harveyi* and *Vibrio cholerae* have been studied extensively, and genomes of multiple strains of each species are available. The genomes of *Vibrio harveyi* BAA-1116 and *Vibrio cholerae* O1 El Tor N16961 were used as reference genomes to identify other possible QS genes contributing to andrimid production in OCN008. To investigate the individual possible pathways identified, the histidine kinase of each was knocked out and the resulting mutants were assessed for andrimid production. In this experiment andrimid production was screened based on the ability of conditioned, cell-free liquid media to

inhibit the growth of the target bacterium *Pseudoalteramonas* sp. strain OCN003. OCN003 was used in these experiments because, like OCN004, it is found readily on *M. capitata* but does not form clumps when grown in liquid culture as OCN004 does, making it more amenable to liquid media assays and spectrophotometric analysis.

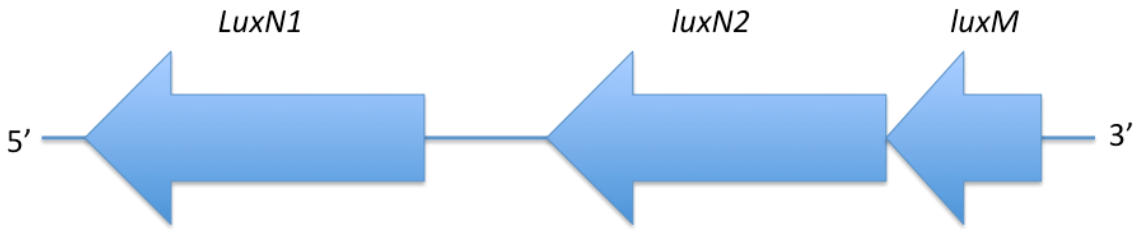

Figure 2.5. Configuration of the two genes annotated as luxN in OCN008. *luxN1* shared the most homology to the *Vibrio harveyi luxN* and was the first one identified as *luxN1*. *luxN2* shared high enough homology that it was also looked as a possible *luxN* gene.

Table 2.3. Percentage identity at the amino acid level of *Vibrio coralliilyticus* OCN008 potential QS genes to the well-characterized Vibrio harveyi and Vibrio cholerae QS genes. **V. cholerae* O395 was used since *V. Cholerae* O1 El Tor N16961 has a naturally occurring frame shift in *hapR*. ** *V. coralliilyticus varS* was found through the RAST server but could not be found in NCBI.

Possible OCN008 QS genes	**Percentage Identity to *Vibrio harveyi* BAA-1116**	**Percentage Identity to *Vibrio Cholerae* O1 El Tor N16961**
aphA (ERB64015.1)	88% (YP_001443333.1)	87% (NP_232275.1)
cqsA (ERB66061.1)	60% (YP_001448208.1)	63% (NP_232914.1)
cqsS (ERB66059.1)	49% (YP_001448209.1)	53% (NP_232913.1)
luxM (ERB62645.1)	31% (YP_001445946.1)	N/A
luxN1 (ERB62643.1)	48% (YP_001445947.1)	N/A
luxN2 (ERB62642.1)	44% (YP_001445947.1)	N/A
luxO (ERB62963.1)	89% (YP_001446139.1)	88% (NP_230666.1)
luxU (ERB62962.1)	54% (YP_001446138.1)	61% (NP_230667.1)
luxP (ERB63170.1)	67% (YP_001447483.1)	65% (NP_233124.1)
luxQ (ERB63172.1)	56% (YP_001447484.1)	59% (NP_233123.1)
luxS (ERB62567.1)	87% (YP_001446656.2)	88% (NP_230208.1)
luxT (ERB66350.1)	84% (YP_001447371.1)	74% (NP_233302.1)
hapR (ERB64458.1)	85% (YP_001446631.1)	76% (YP_002818855.1)*
csrA (ERB62341.1)	98% (YP_001446672.1)	97% (NP_230199.1)
varA (ERB62638.1)	95% (YP_001445929.1)	90% (NP_230858.1)
*varS***	77% (YP_001446693.1)	74% (NP_232082.1)

Homologues to 10 additional protein coding genes were identified via BLAST: *cqsA*, *cqsS*, *csrA*, *luxM,* a second *luxN, luxO, luxQ, luxS, luxU, VarA*. Table 2.3 shows the percent

identity at the amino acid level of OCN008 possible QS genes to the characterized genes of *Vibrio harveyi* and *Vibrio cholerae*. The genome of OCN008 showed two genes annotated as *luxN*. The orientation of these two genes relative to *luxM* is shown in figure 2.5. The *luxN1* gene was investigated first because it had been hit in our initial transposon screen, and was named *luxN1*. Due to the orientation of ERB62642.1 (*luxN2)*, and its homology to the *Vibrio harveyi luxN*, it was chosen for study as *luxN2.* The genes encoding histidine kinases *cqsS*, *luxN2* and the region encompassing both *luxN1* and *luxN2* were deleted and assayed for andrimid production. To assess the role of the VarS/A pathway, the response regulator *varA* was chosen for study because, in the publication that identified the pathway, its deletion yielded a 10-fold greater reduction in the QS response than the deletion of the histidine kinase *varS*[86]. Mutations were made as described above.

All mutants showed andrimid production similar to that of the WT except for the Δ*luxO* and Δ*varA* mutants, which both produced andrimid, but less than that of the wild type (Fig. 2.6A). The Δ*varA* mutant produced even less andrimid than the Δ*luxO* mutant, with target strain growth observed when conditioned media was only diluted 1:2. The decreased andrimid production displayed by these mutants was only detectable when the liquid media assay described here was used. To verify that the mutations screened previously using the solid media assay did not cause an undetectable decrease in andrimid production, the mutants corresponding to the genes implicated by the transposon screen were assessed for andrimid production using the liquid media assay. The results showed, as previously, that the Δ*aphA* and Δ*luxN1,* produced andrimid in the same manner as the wild type, while the Δ*hapR,* Δ*luxP* and Δ*luxT* mutants

produced no detectable andrimid (Fig. 2.6B). In these experiments, a strain lacking the andrimid biosynthesis operon (OCN041) was used as a negative control for andrimid production.

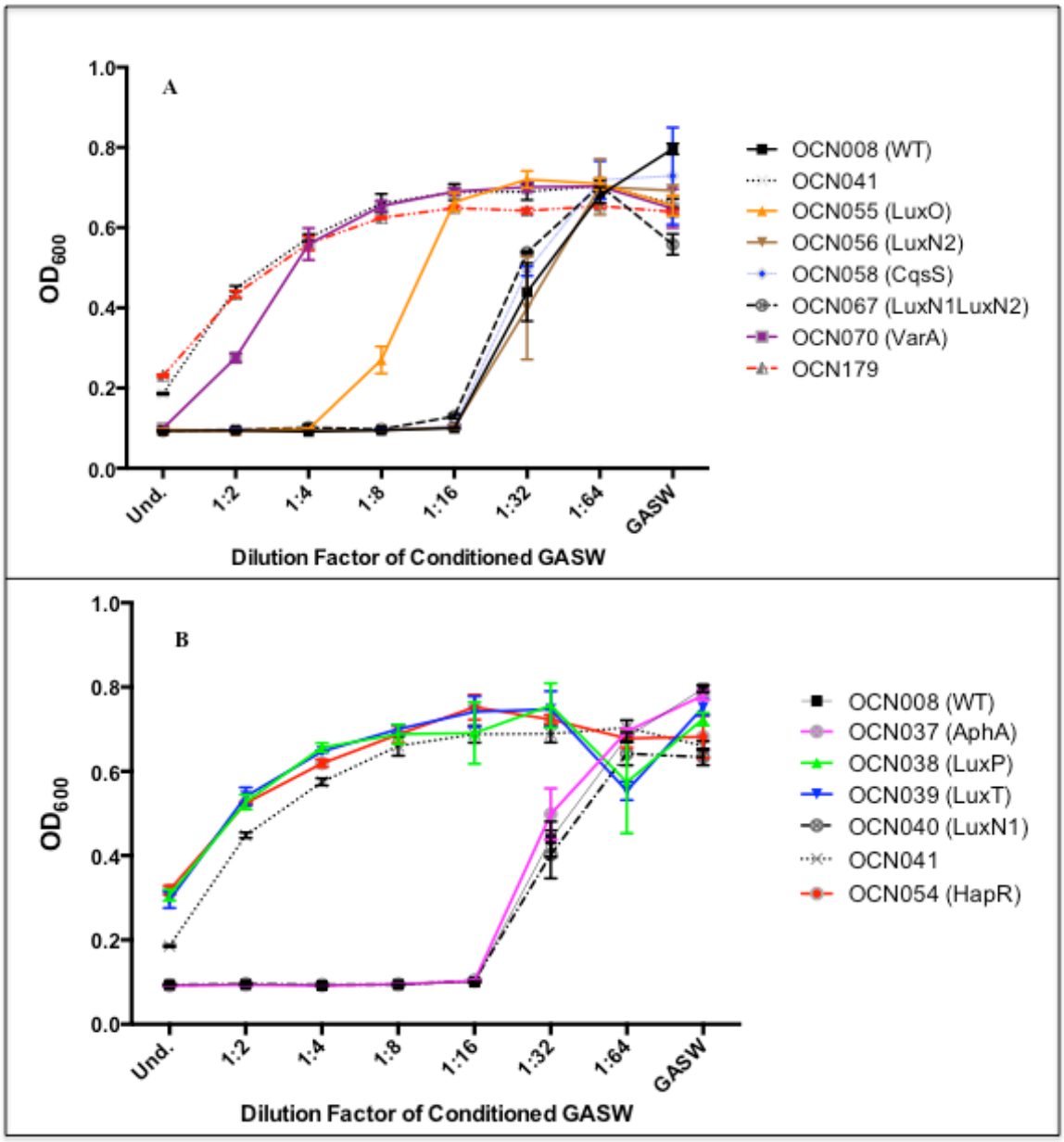

Figure 2.6. Growth of the target bacterium OCN003 in different dilutions of cell-free conditioned media from **(A)** wild type OCN008, OCN041 (Δ*adm* – Andrimid operon mutant that produces no andrimid), OCN055 (Δ *luxO*), OCN056 (Δ *luxN1*), OCN067 (Δ *luxN1* Δ *luxN2*), OCN058 (Δ *cqsS*), OCN070 (Δ *varA*) and OCN179 (*luxO(D47E))*, and **(B)** OCN008, OCN037 (Δ*aphA*), OCN038 (Δ*luxP*), OCN039 (Δ*luxT*), OCN040 (Δ*luxN2*), OCN041 and OCN054 (Δ*hapR*).

HapR and andrimid production are regulated through LuxO

In *V. harveyii* and *V. cholerae* a single amino acid mutation from an aspartic acid to a glutamic acid at position 47 (LuxO(D47E)) causes LuxO to take on a conformation that mimics the phosphorylated form on LuxO, locking the cell into a low cell density behavior since the qrrs are constantly expressed[91,135]. To determine if LuxO was involved in QS in OCN008 in a similar fashion, a chromosomal *luxO(D47E)* mutation was constructed and screened for andrimid production. This mutant behaved exactly like the HapR mutant (figure 2.6A), suggesting that HapR in OCN008 is indeed regulated via LuxO in a similar fashion to other *Vibrios.* It should be noted that while LuxO appears to be central to QS regulation of andrimid production, the LuxO mutant (a situation where HapR should be present at high levels even at low cell density) produced less andrimid than the wild type, suggesting that QS regulation of andrimid production is not as simple as some QS outputs (for example in *V. harveyi* more LuxR – the HapR homologue – equals more bioluminescence)[136].

Abrogation of andrimid production is not the result of a growth defect.

To address the possibility that the abrogation of andrimid production seen in the mutants was not an artifact of impaired growth, growth curves were performed using a 96-well microplate and a plate reader. All mutants tested grew at the same rate and to the same OD as wild type OCN008 (Figure 2.7).

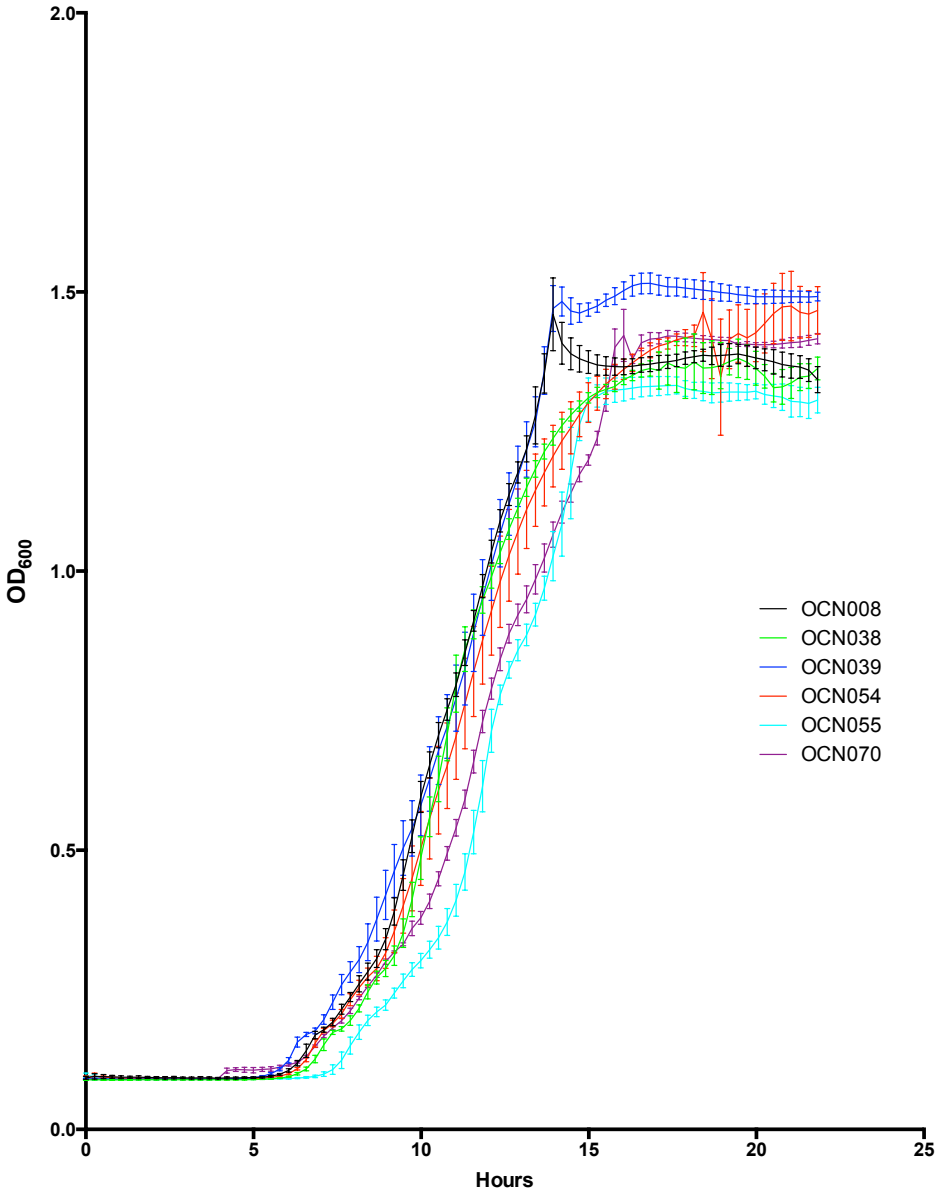

Figure 2.7. Growth curves of wild type OCN008 and andrimid deficient mutants. OCN037 = Δ a*phA*, OCN038 = Δ *luxP*, OCN039 = Δ *luxT*, OCN054 = Δ *hapR,* OCN055 = Δ *luxO*, OCN070 = Δ *varA*. Growth was monitored for 24 hours with time points taken every 15 minutes using an automated 96 well plate reader.

Addition of boric acid to media increases andrimid production

Boron is a component of the AI-2 molecule, and its addition to growth medium has been proposed to increase production of AI-2 in *V. harveyi*[137,138]. Because deletion of *luxP* reduced andrimid production,andrimid levels were screened after growth in medium supplemented with boron (to cause elevated AI-2 levels) to further investigate the role of the AI-2 LuxPQ/S system in andrimid production. When GASW media was supplemented with 0.4% boric acid, andrimid production increased. Conditioned GASW inhibited the growth of the target bacterium OCN003 only until a dilution of 1:2 (Fig. 2.8). However, when GASW was supplemented with 0.4% boric acid, OCN003 growth was not observed until the conditioned media had been diluted 1:32 and above, indicating increased andrimid production in the boron containing media.

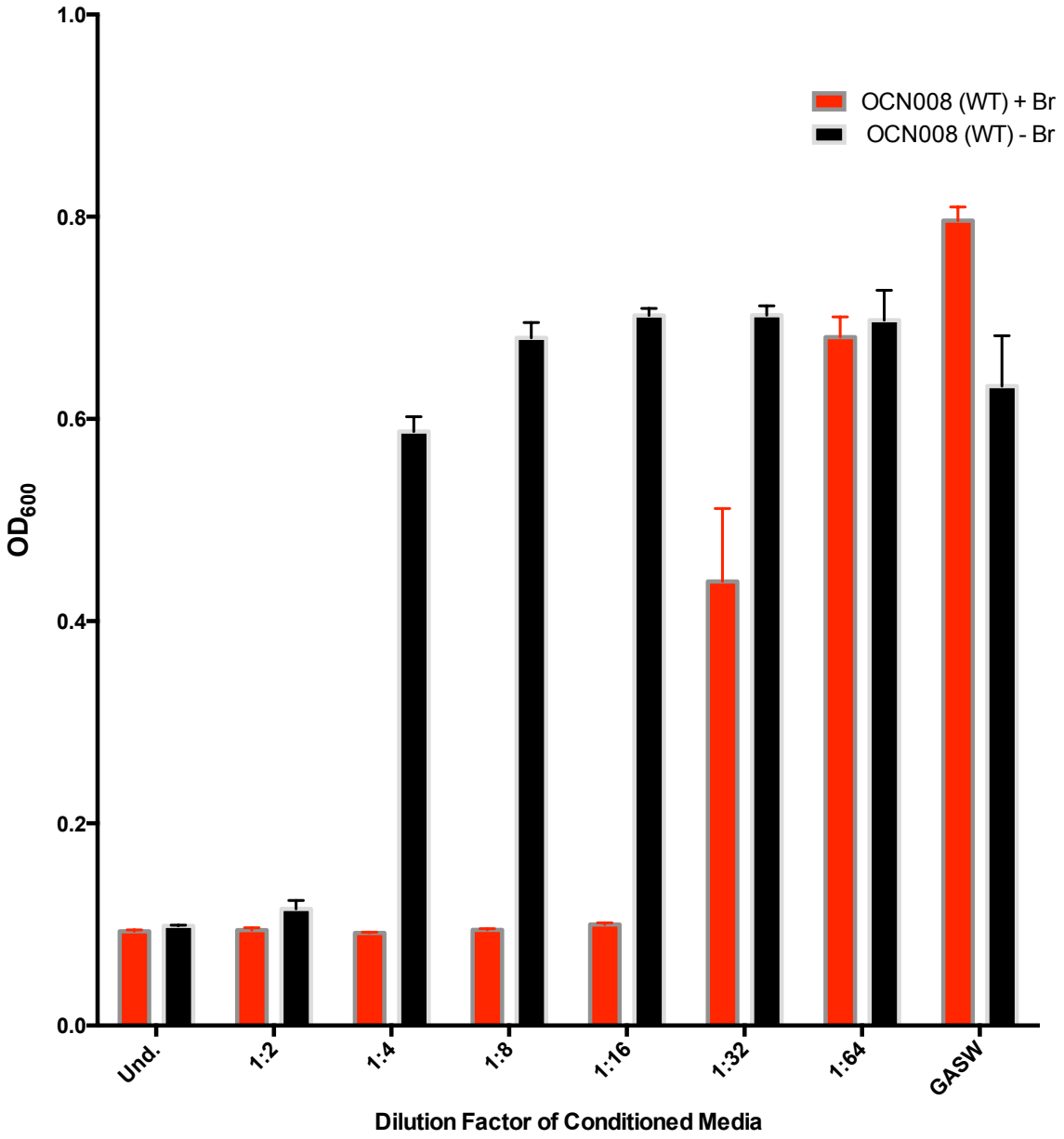

Figure 2.8. Growth of the target bacterium OCN003 in different dilutions of cell-free conditioned media from wild type OCN008 grown in GASW (black lines) and GASW supplemented with 0.4% Boric Acid

DISCUSSION

In this work, both classical and reverse genetic approaches were used to determine which QS genes / pathways contribute to the regulation of andrimid production in *Vibrio coralliilyticus* strain OCN008. It was found that andrimid production in OCN008 requires the genes *luxP*, *hapR*, and *luxT*. Additionally, a fully functional *luxO* is required, and *varA* contributes but is not necessary. This suggests that OCN008 employs two QS pathways, the LuxPQ/S and VarS/A systems, to regulate andrimid production via the high cell density QS master regulator HapR. Additionally, LuxO was shown to be central to QS activation in a manner similar to other described *Vibrios*. While quorum sensing has been implicated in the virulence of *Vibrios* in humans and other organisms, this represents the first link between QS and virulence in the field of coral disease. Given the poor understanding of coral disease presently, any insight into mechanisms of virulence provides new opportunity to understand how reefs are lost and, hopefully, gets closer to mitigating the problem. Additionally, andrimid has been shown to be a novel virulence factor – no other pathogenic organism produces and uses an antibiotic as a virulence factor – and therefore represents a new infectious paradigm that warrants study.

One possible explanation for the mutants' described above inability to produce andrimid is that the gene deletion caused a growth impairment that prevents the strain from ever reaching quorum levels of cell density. For example, in the transposon screen that implicated QS, the cyclic-AMP receptor protein (CRP) was knocked out, resulting in an andrimid null phenotype. Further investigation showed, however, that this mutant (as well as a CRP clean deletion mutant) reaches stationary phase at an OD600 of ~1.0 (using a 1cm path-length) as opposed to the wild type which reaches stationary phase at an OD600 of ~4.0. Andrimid production is not detectable until the wild type reaches an OD of ~2.8. In this case, the growth impairment rendered by

disruption of CRP function explains the andrimid null phenotype, rather than suggest that CRP regulates andrimid production. This was not the case for the mutants screened here, as they all grew in manner comparable to the wild type.

The decreased andrimid production displayed by the Δ*luxO* mutant is notable because, if the QS circuit in OCN008 is similar to other described systems, a Δ*luxO* mutant should have higher levels of the master regulator HapR at low cell densities than the wild type. This finding was contrary to the initial hypothesis when justifying creation of Δ*luxO*. It was hypothesized that this mutant would produce more andrimid than the wild type since, in theory, HapR would be present at an earlier point in the growth phase and, as discovered above, is required for andrimid production. This turned out not to be the case, as the target strain began to grow in mutant-conditioned medium diluted 1:8, as opposed to the wild type, where target strain growth was not observed until the conditioned media had been diluted 1:32. This implies that HapR does not directly regulate the andrimid operon, or that HapR dynamics are different in OCN008 relative to other *Vibrios*.

The AI-2 pathway was shown to be the most important QS pathway in regards to andrimid production. This pathway was shown to be necessary, and the addition of boron to the medium caused an increase in andrimid production, consistent with an AI-2 dominated QS response. The boron containing AI-2, the auto-inducer for the LuxPQ/S pathway, is a furanosyl borate diester. The synthase of this molecule is the protein LuxS, although LuxS actually produces the pre-curser molecule to AI-2, 4,5-hydorxy-2,3-pentadione (DPD), which then undergoes a spontaneous reaction with water and boric acid to ultimately form the boron containing AI-2 molecule[139]. The product of the spontaneous reaction is either the boron-containing molecule mentioned above, or the non-boronated R-THMF (2R,4S-2-methyl-2,3,3,4-

tetrahydroxytetrahydrofuran)[139]. The direction of the spontaneous reaction is determined by the amount of boron present. Since LuxP is required for andrimid production and is known to bind AI-2, the increase in andrimid production upon addition of boric acid to the media is highly suggestive that QS in OCN008 is predominantly AI-2 mediated [137].

Often times the AI-2 pathway is thought of as an inter-species communicative molecule due to diverse subsets of organisms found capable of producing and detecting AI-2 (including most *Vibrios*)[140,141]. This would represent, however, the first *Vibrio* that appears to rely *solely* on AI-2 molecules to activate QS. In every other case, at least one species specific AI is employed, necessitating same species quorums to initiate QS. This would suggest that OCN008 may not need to reach a quorum itself to initiate its QS system, but rather, activates QS if quorum levels of any AI-2 producing bacteria are reached. In one study, that examined the QS molecules that naturally occur in the mucus layer of coral tissue, it was discovered that AI-2 was present in every coral at sample location. It has also been estimated that roughly 90% of marine bacteria are gram-negative rods, a group found to possess homologues to LuxS nearly ubiquitously.

Research has shown that coral mucus contains ~10^5 to 10^6 CFUs/mL of bacteria, a number too low to activate QS in OCN008[142]. If QS is required for infection this may explain why OCN008-like *V. coralliilyticus* bacteria can be found in the environment around *M. capitata* even when outbreaks are not occurring and in areas where no disease signs are present[100]. In other words, under normal, healthy conditions, OCN008 is unable to infect because two few bacteria are present to induce QS and resources are too limited and/or competition is too extreme to allow for proliferation to quorum levels. However, if any perturbations occur that result in an increase in the level of AI-2 producing bacteria, or that damage the healthy coral microbiota, QS can be activated and andrimid (and possibly other virulence factors) are expressed, and infection

begins. Such a scenario would have implications in areas such as Kaneohe Bay, an area in Hawaii relatively rich in *M. capitata* coverage, where sewage spills and nutrient enrichment are not uncommon[34,143]. In the event of a sewage spill, high numbers of bacteria are deposited into the ocean, and during nutrient enrichment events fast growing heterotrophs proliferate quickly. In areas such as South Kaneohe Bay (the location of Coconut Island) where water circulation is limited[144], this potentially translates to periods of increased numbers of water column and coral mucus associated AI-2 bacteria. Perhaps this explains why the most serious outbreaks of aMWS have occurred after heavy rain events (heavy rain-water / sewage runoff events).

This work provides an entry point to at least one area of aMWS preventative or treatment research. QS inhibition has been used successfully to prevent and cure certain infectious diseases. As previously mentioned, QS has been used to cure and prevent Potato Soft Rot and Tomato Crown Gall, infectious diseases detrimental to the agriculture industry. These infections, caused by the bacteria *Erwinia cartotovora* and *Agrobacterium tumefaciens,* respectively, utilize an AHL mediated QS system to facilitate infection. Disease prevention was achieved after a gene capable of degrading AHLs was introduced to the commensal rizosphere bacterium *Pseudomonas fluorescens* P3, rendering the strain capable of preventing QS activation. Additionally, QS inhibition has been used to prevent some biofilm dependent *Staphylococcal* infections. As QS continues to be a major target of study for disease prevention in other systems, QS modulation may be used to mediate coral disease outbreaks, but only if the research continues to be supported.

Table 2.1. Strains and Plasmids used in this study

Vibrio Coraliillyticus Strains	**Relevant Characteristics ***	**Source or Reference**
OCN008	Wild Type; Ap^R	[61]
OCN037	Δ*aphA;* Ap^R	This Study
OCN038	Δ*luxP;* Ap^R	This Study
OCN039	Δ*luxT;* Ap^R	This Study
OCN040	Δ*luxN1;* Ap^R	This Study
OCN041	Δ*adm* operon*;* Ap^R	[99]
OCN054	Δ*hapR;* Ap^R	This Study
OCN055	Δ*luxO;* Ap^R	This Study
OCN056	Δ*luxN2;* Ap^R	This Study
OCN058	Δ*cqsS;* Ap^R	This Study
OCN067	Δ*luxN1*Δ*luxN2;* Ap^R	This Study
OCN070	Δ*varA;* Ap^R	This Study
OCN138	Complemented Δ*luxT* strain; Ap^R,Cm^R	This Study
OCN139	Complemented Δ*luxP* strain; Ap^R,Cm^R	This Study
OCN149	OCN039 w/ empty pBU115; Ap^R,Cm^R	This Study
OCN150	OCN038 w/ empty pBU115; Ap^R,Cm^R	This Study
OCN151	OCN054 w/ empty pBU115; Ap^R,Cm^R	This Study

OCN152	Complemented Δ*hapR* strain; Ap^R,Cm^R	This Study
OCN179	*luxO(D47E);* Ap^R	This Study
Marine Bacterial Strains		
OCN003	*Pseudoalteramonas* sp. sensitive to andrimid	This Study
OCN004	*Alteramonas* sp. sensitive to andrimid	This Study
E. Coli Strains		
SM10λPir	F+ conjugation strain; Km^R	[145]
β3914	DAP Auxotroph; Km^R	[122]
π3813	Thymidine Auxotroph; Em^R	[122]
Plasmids		
pBlueScript SK+	Cloning vector; Ap^R	Stratagene
pBT20	Mariner Transposon containing suicide vector; Gm^R, Ap^R	[146]
pRK2013	Self Transmissible plasmid; Km^R	[147]
pSW4426T	Suicide Vector used to make	[122]

	deletion plasmids; Cm^R, Sp^R, Sm^R	
pEVS78	Replicative vector that can be maintained by OCN008; Cm^R	[148]
pBU115	pEVS78 w/ unique *Sma*I site in the MCS; Cm^R	[99]
pAHB102	pSW4426T used to make OCN054; Cm^R, Sp^R, Sm^R	This Study
pAHB103	pSW4426T used to make OCN055; Cm^R, Sp^R, Sm^R	This Study
pAHB104	pSW4426T used to make OCN056; Cm^R, Sp^R, Sm^R	This Study
pAHB105	pSW4426T used to make OCN058; Cm^R, Sp^R, Sm^R	This Study
pAHB123	pSW4426T used to make OCN067; Cm^R, Sp^R, Sm^R	This Study
pAHB124	pSW4426T used to make OCN070; Cm^R, Sp^R, Sm^R	This Study
pAHB180	pEVS78::luxP region - LuxP complement vector; Cm^R	This Study
pAHB181	pEVS78::luxT region - LuxT complement vector; Cm^R	This Study
pAHB183	pSW4426T used to make	This Study

	OCN179; Cm^R, Sp^R, Sm^R	
pAHB189	Arabinose inducible replicative expression vector; Cm^R	This Study
pAHB190	pAHB189::HapR CDS - HapR complement vector; Cm^R	This Study
pPJAV250	pSW4426T used to make OCN037; Cm^R, Sp^R, Sm^R	This Study
pPJAV253	pSW4426T used to make OCN040; Cm^R, Sp^R, Sm^R	This Study
pPJAV254	pSW4426T used to make OCN038; Cm^R, Sp^R, Sm^R	This Study
pPJAV255	pSW4426T used to make OCN039; Cm^R, Sp^R, Sm^R	This Study

*Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Gm^R gentamycin resistance; Sp^R, spectinomycin resistance; Sm^R, streptomycin resistance.

Table 2.2. Primers used in this study

Primers	**Sequence**
aphA-up-	ATATAGGATCCCACGTTACTATGATGAGCGCTTAGCTG

BamHI-F	
aphA-up-SmaI-R	GAGTACTTCCCCGGGTAATGACATGTCTTCAATCCAAATGGTC
aphA-dn-SmaI-F	ATGTCATTACCCGGGGAAGTACTCGCTGAATTAGCCTCTCTAGGC
aphA-dn-NruI-R	GTATGGCAAGCCATTGTTCGCGAAGC
aphA-outside-F	CGTGAGTGGCCGTTCTTCTCCACTCGC
aphA-outside-R	CACTTAAGCAAGCTTAGGTGAAGGGTTGAG
hapR-up-EcoRI-F	ATATAGAATTTCCTCACGACGAGACGGCTTATCAAGCAGCGTAC
hapR-up-SmaI-R	TAGGTAGCTCCCGGGACGAGCAAACACCTCTAATGCGATTTCCATC
hapR-dn-SmaI-F	TTTGCTCGTCCCGGGAGCTACCTAGATATGCTTTGCATCTACAAG
hapR-dn-EcoRI-R	ATATAGAATTCGACGGTATCTACGTGTCAATGGAAGGCAAG
hapR-outside-F	CTGCGCGTAATCAATACCCACACCAACAAC
hapR-outside-R	GATATCGTTAAAGTTTACACTAAGCGTGTG
HapR-NdeI-F	ATATACATATGGATTCTATAGCTAAGAGACCGCG
HapR-SalI-R	ATATATGTCGACCTACTTGTAGATGCAAAGCATATC
luxN1-up-BamHI-F	ATATAGGATCCGGGCTAGGATTAGGATATTGCCAGCGTG

luxN1-up-SmaI-R	CGATGTCAGCCCGGGTGAAACCTGATCTAACATATAGCTGAACATAATG
luxN1-dn-SmaI-F	CAGGTTTCACCCGGGCTGACATCGACTTTGCCAGCTACGATGCC
luxN1-dn-SacI-R	ATATAGAGCTCGAATTGCTGTCGCTACGATGAGAG
luxN1-outside-F	CCATTAAGCGAGAGCGGTAGCAAGAGC
luxN1-outside-R	GCCACCATCAAGAAGGTGACAAGCTGC
luxN2-up-EcoRI-F	ATATAGAATTCATCTTGCTTTCAAATATCGAATTATTTGTCATC
luxN2-up-SmaI-R	GAAGTAAGCCCGGGGTTACTCCCCCAGCTAACTAAATACGGAGAGTAG
luxN2-dn-SmaI-F	GGAGTAACCCCGGGCTTACTTCTTCTTTACCTGTAACTGCGCATTAAGC
luxN2-dn-EcoRI-R	ATATAGAATTCAACAACAGCACTTTGCCAATGTCCAATAG
luxN2-outside-F	CATTGAGTATTACAGTGACCTGATCGAAG
luxN2-outside-R	GTGAATCCGAGCTGTATAATCACGGTTG
luxO-up-MunI-	ATATACAATTGCAGGTTCCGTTGTCCAAGGTCGCAGAGC

F	
luxO-up-SmaI-R	AGCTTTCTTCCCGGGGTCCGCTTCACACGGTTTGATAAGGAAATCCTG
luxO-dn-SmaI-F	GAAGCGGACCCCGGGAAGAAAGCTATTGAGCAAGCGATTCAG
luxO-dn-MunI-R	TATATCAATTGTTCCGGCGACCAGTGAAGGTGTGCTCGCG
luxO-outside-F	CGATAGTGTAACGAAATCTATGCACAAGGCG
luxO-outside-R	TGGATGCCATCAACCTTATCCGCAACATGC
luxO(D47E)-F	CCTGATTTAATCCTATTGGAACTTCGTCTTCCCGACATG
luxO(D47E)-R	CATGTCGGGAAGACGAAGTTCCAATAGGATTAAATCAGG
luxO(D47E)-seq	GCAAACACAAAAGTCCAAATATCTTTTG
luxP-up-BamHI-F	ATATAGGATCCAACTCTCGCGTAGACATGATTGC
luxP-up-SmaI-R	TCTCTGCTTCCCGGGCGCCAATGATAACAGCGTTGCTTTAAACATTG
luxP-dn-SmaI-F	TCATTGGCGCCCGGGAAGCAGAGAGCGTTTAGGTACTCGGATC
luxP-dn-SacI-R	ATATAGAGCTCGGTCAGGTCTGTCTTCGATACCATG
luxP-outside-F	CGTTGCTTCGGTTGCGTTACCTCTGAATGC
luxP-outside-R	GTGGCTTCGCATAAGGATCACGATAGATTG
luxT-up-BglII-F	ATATAAGATCTAGCTTAGCGAGCTAGGGAGCGAGTC

luxT-up-SmaI-R	CTTACCAATCCCGGGTTCAGTATCTTCTTTACTACGCTTTGGCATG
luxT-dn-SmaI-F	GATACTGAACCCGGGATTGGTAAGTCACTGGTTCGCATGGCGAAG
luxT-dn-SacI-R	ATATAGAGCTCCTGCAATCAACGAAATGCACTCAAC
luxT-outside-F	GGTTAGGGTCAATAAAGAGATGATGGAGT
luxT-outside-R	ATTGTGAAACCACAGCAGAGAACCTTGTG
varA-up-MunI-F	CAATTGCTAGAGTGTTGAATGCTGCTTTGTC
varA-up-SmaI-R	TTCCATGGCGCCCGGGACCACTTTCAGCTTCCCCTGCTACGTTC
varA-dn-SmaI-F	GCTGAAAGTGGTCCCGGGCGCCATGGAATGTTAGACACCGAG
varA-dn-MunI-R	CAATTGCGTCCATATCATCTTGAGAGTCTTCAG
varA-outside-F	GCGGCATCAGCACCTTGTCGCCTTGC
varA-outside-R	GATGAGCATTCATCAGGCGGGCAACG
araC-ccdB-F	AGCCGTCAATTGTCTGATTCGTTACC
araC-Pbad-NdeI-R	CATATGCGTTTCACTCCATCCAAAAAAACGG

CHAPTER 3: QUORUM SENSING REGULATES VIRULENCE IN THE HAWAIIAN CORAL PATHOGEN *VIBRIO CORALLIILYTICUS* STRAIN OCN008

INTRODUCTION

Coral reefs represent some of the most diverse ecosystems on the planet[1]. Despite harboring marine life that has yielded millions in tourism and fisheries dollars, provided new medical discoveries and housed some of the highest rates of primary production on the planet, reefs are under constant threat[149,150]. Climate change, overfishing, improper tourism practices and disease have all contributed to reef damage over the past 30 years[151]. In the past 10-20 years, coral disease has emerged as one of the more serious threats to reef ecosystems. In particular, tissue-loss diseases have presented a major problem in areas such as the Caribbean and the Indo-Pacific. Research has identified most tissue loss diseases to be the result of infectious pathogens[5]. Due to the nature of the spread of infections, pathogen-induced tissue-loss disease can leave large stretches of reef lifeless, with nothing but coral skeletons remaining. This has multiple implications: 1) corals are slow growing animals making recovery from diseases and 2) the coral's bare skeleton eventually crumbles leaving reef surfaces unsuitable for coral larval settlement, leading to little hope that reefs will be capable of rebuilding[152–154].

Despite the realization that the most severe outbreaks of coral disease are typically pathogen induced, few pathogens have been identified[5]. Of those identified, bacterial pathogens have been the most common[5]. Fortunately, research on coral disease is increasing and many of the pathogens have been identified in the past 10 years. To study coral disease, researchers have borrowed strategies used by those who study human diseases. The goal, of course, is to find ways to prevent or cure coral diseases once causative agent(s) have been found. In order to do

so, specific characteristics about the pathogen must be uncovered, as pathogen ID is usually insufficient. After pathogen identification, researchers often look for mechanisms that facilitate infection, which provide good targets for preventative strategies. While the rate at which coral pathogen identification has increased, the underlying mechanisms of virulence remains poorly understood.

Recently a strain of *Vibrio coralliilyticus* was identified as a causative agent of the disease Acute *Montipora* White Syndrome (aMWS), a rapidly progressing tissue-loss disease affecting the Hawaiian reef building coral *Montipora capitata* (Figure 3.1) in Kaneohe Bay, Oahu[61]. While *M. capitata* diseases have been found in Hawaii for over 10 years, two major outbreaks of aMWS, affecting over 300 and over 1200 colonies of *M. capitata,* occurred in 2010 and 2012, respectively, demonstrating aMWS as a serious threat in Kaneohe Bay in need of study[23, 59,155]. *Vibrio coralliilyticus* strain OCN008, hereafter referred to as OCN008, induced aMWS signs in a laboratory setting in 80-90% of inoculated coral fragments. Interestingly, OCN008 produces and uses the antibiotic andrimid as a virulence factor[99]. In the previous chapter, an investigation into andrimid regulation revealed quorum sensing as necessary for andrimid production. This represented the first regulatory network demonstrated to be involved in the pathogenesis of a coral disease-causing agent, albeit indirectly, as QS was only shown to regulate a known virulence factor.

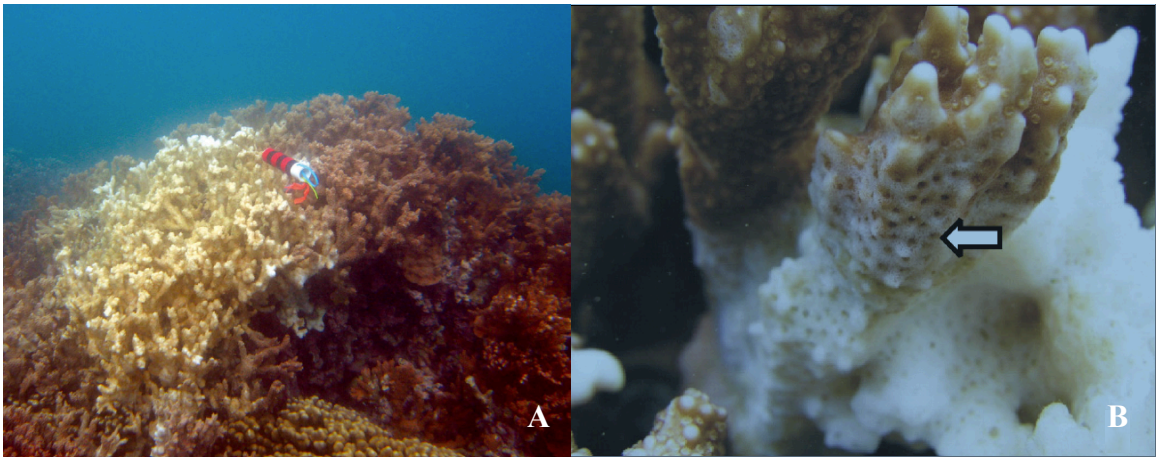

Figure 3.1. Acute Montipora White Syndrome. A) A colony of *Montipora capitata* exhibiting signs of aMWS. Note the exposed white skeleton in the center of the colony. Bars on the scale bar represent 1cm. **B)** A close up of an aMWS affected coral branch. Exposed holes remain where polyps that have lysed previously resided. At the legion front, remnants of tissue remain (white arrow) as the legion progresses.

Quorum sensing (QS) is a mechanism that allows for bacteria to exhibit distinctly different behaviors based on cell density[156]. It is thought to have evolved because bacteria derive certain benefits from group behaviors otherwise impossible to achieve by small populations acting alone. QS in the genus *Vibrio* was first described to regulate bioluminescence in *Vibrio fisherii* and *Vibrio harveyi*[113,157]. Since then *V. harveyi* and the human pathogen *V. cholerae* have become model systems for QS in *Vibrios.* The representative QS circuits from *V. cholerae* and *V. harveyi* are shown in figure 3.2. The switch from low cell density (LCD) to high cell density behaviors (and visa-versa) is dependent on the concentration of extracellular autoinducers, small molecules produced and excreted at constant rates by individual cells, and ultimately the phosphorylation state of the response regulator LuxO[158]. Phosphorylation of LuxO occurs via multiple histidine kinase receptors acting in parallel[84,136]. At LCD, autoinducer (AI) concentration is low, inducing histidine kinase receptor autophosphorylation which results in a phosphorylated LuxO. The phosphorylated form of LuxO is an active transcriptional

activator whose primary responsibility is inducing the transcription of regulatory RNAs (Quorum Sensing

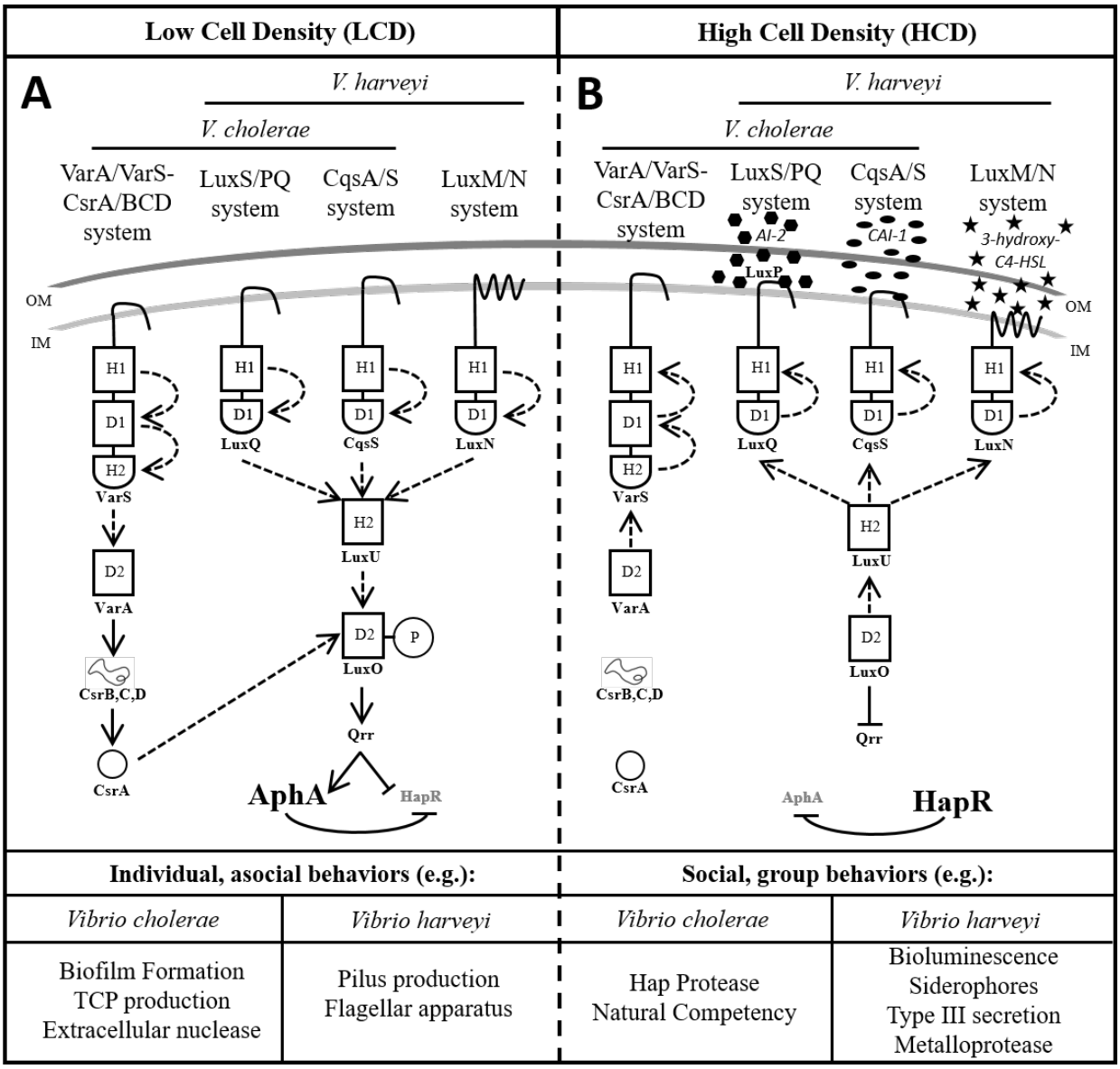

Figure 3.2. Canonical quorum sensing circuits of Vibrio harveyi Vibrio cholerae. **A)** Low cell density behavior. At low cell densities, histidine kinase sensors are unoccupied and the phosphate flow moves towards LuxO. The phosphorylated LuxO induces transcription of the QS regulatory RNAs (qrrs), which, in turn, aid in the expression of the LCD master regulator AphA and directly lead to the degredation of the HapR mRNA. **B)** High cell density behavior. Once a high enough cell density is achieved, histidine kinase receptors are occupied and the phosphate flow reverses, leading to an inactive, un-phosphorylated LuxO. This quickly leads to loss of qrrs, decreases in AphA levels, increasing HapR levels and the HCD response.

Regulatory RNAs - Qrrs)[159]. When Qrr transcription is active, the master regulator of LCD behavior AphA is active, and the mRNA of the HCD master regulator HapR is actively degraded[89]. Group behaviors are achieved when cell densities reach quorum levels, leading to increased concentrations of AIs. Once the AI concentration is high enough such that the majority of AI-specific histidine receptors are occupied, the phosphorylation flow is reversed, and LuxO loses phosphorylation and becomes transcriptionally inactive. Without the transcription of the qrrs, AphA levels drop and HapR levels increase, leading to a shift in the expression profile of the group. The individual genes expressed at low or high cell density are determined by the presence of AphA or HapR, respectively, and in some cases both[90].

Quorum sensing's association with virulence in *Vibrios* has made it a target of study for decades[160]. In that time, many QS controlled virulence factors have been discovered such as hemolysins, extracellular proteases, lipases, DNAses, biofilm formation, type III and type IV secretion systems and many more[90, 118, 126, 158,160–165]. Because QS was found to regulate the virulence factor andrimid production in OCN008, this work aimed to identify other QS regulated phenotypes in OCN008 that have been shown to be associated with virulence in other *Vibrios.* In the previous chapter, a given gene's involvement in QS was judged based on a mutant's ability to produce andrimid. As andrimid is not a traditional QS product, these screens may uncover other genes contributing to QS in OCN008 missed when assessing QS based on andrimid alone. Specifically, hemolysin, protease, lipase, and biofilm formation will be assessed. Finally, to determine the extent to which QS is involved in the pathogenesis of OCN008, the contribution to virulence of each gene found to be involved in QS was assessed using *in-vitro* infection assays with *M. capitata* fragments.

MATERIALS AND METHODS

Bacterial strains growth conditions

Strains used in this study are listed in table 3.1. Unless otherwise noted, all *Vibrio* strains were grown in GASW medium at 28°C[121]. For solid medium, 15 g L^{-1} of Agar was added prior to autoclaving. Blood agar plates (BAPs) were made from tryptic soy agar (Difco) supplemented with 10 g L^{-1} NaCl and 5% sheep blood. Skim milk agar (SMA) plates were made with skim milk powder (Difco) per the manufacturers recommendations and supplemented with 20 g L^{-1} NaCl and 15 g L^{-1} agar.

Hemolysin assays

Extracellular hemolysin production was screened using BAPs[166]. Strains were grown in liquid GASW overnight, diluted 1:100 and, streaked or spotted (10 μL) onto BAPs. Results were interpreted qualitatively as α, β, or γ hemolysis. Experiments were replicated at least three times ($n \geq 3$), and the figure shown represents one trial. No differences were seen between any replicates.

Protease assays

Extracellular protease assays were performed using SMA[167]. Cultures were grown overnight in GASW, streaked to isolation, and individual colonies were patched onto SMA plates. Results were interpreted qualitatively as positive or negative for protease production based on the presence/absence of SMA zones of clearing. Experiments were repeated at least three times with no inconsistencies.

Biofilm assays

Biofilm assays were performed using the protocol described by O'toole et. al[168]. Strains were grown overnight in GASW, diluted 1:100, and inoculated in quadruplicate into 100 µL of GSW medium (0.22µ membrane filtered sea water supplemented with 2 g L^{-1} yeast extract, 4 g L^{-1} tryptone and 2 mL L^{-1} glycerol) in a round bottom 96-well plate. The plate was incubated standing at 28°C for 24 hours, then the plate was washed three times with mili-Q water. Next, 125 µL of 1% crystal violet (in water) was added to each well and incubated for 10 minutes. The plate was washed three times with water, and 125 µL of 30% acetic acid was added to each well. After 15 minutes of incubation, 100 µL from each well was transferred to a new, flat bottom 96 well plate and the optical density was read at 550 nm (OD_{550}). Biofilm assays were repeated 3 times, with no significant differences between any replicates.

Coral collection and laboratory infection trials

Fragments of *Montipora capitata* measured approxamitely 3 cm x 3 cm and were collected from a fringing reef (a reef where *M. capitata* is one of the dominant coral species[60]) surrounding Moku o Loʻe in Kaneʻohe Bay, Oʻahu, Hawaiʻi under permit 2015-48 granted by the Hawaiʻi Department of Aquatic Resources. Fragments were allowed to recover for 3 days in flow through water tables before the onset of experimentation.

Coral fragments were allowed to acclimatize to 27°C in aquaria (infection trial temperature) over the course a day prior to inoculation. Filtered seawater (FSW – seawater passed through a 0.22 µm-pore-size filter) was used for all laboratory trials. Trials were set up using a block design as previously described[24]. Briefly, coral fragments were placed on elevated

plastic grates in plastic aquaria (3L). Air pumps were used to achieve water circulation and water was changed every 5 days. Corals were held under ambient light at 27°C and monitored daily for signs of tissue loss. Fragments within each replicate came from the same coral colony, and different colonies were used for each individual replicate. Two negative controls were used: a FSW inoculated control and a bacterial control - a fragment inoculated with *Alteramonas* sp. strain OCN004, an isolate frequently cultured from the mucus of *M. capitata*).

Infection trial inocula were prepared as follows: strains were grown in GASW overnight, diluted to an OD600 of ~0.05 in fresh GASW, and grown at 27°C until an OD600 of 1.6 was reached. Cells were then washed three times and then re-suspended with FSW. Tanks were inoculated to a final concentration of 10^8 CFU L^{-1} tank of water. Bacterial inocula were pipetted directly over each fragment with care not to damage or disturb the fragment. Corals were monitored daily and experiments ran for a maximum of 14 days.

RESULTS

Quorum sensing positively regulates β-hemolysin and extracellular protease production but does not regulate lipase production in Vibrio coralliilyticus strain OCN008.

Vibrio coralliilyticus strain OCN008 and mutants were screened for hemolysin and extracellular protease production using Blood Agar Plates (BAP) and Skim Milk Agar Plates (SMA), respectively. OCN008 produces a β-hemolytic reaction on a BAP in roughly 48 hours, and shows proteolytic clearing on SMA in about 24 hours. All mutants screened produced similar hemolytic and proteolytic reactions to the wild type except for the Δ*hapR* and Δ*luxP* mutants, which displayed α-hemolysis but never β-hemolysis on BAPs and, never showed any clearing on the SMA (figures 3.3 and 3.4). Interestingly, the Δ*luxT*, Δ*luxO* and Δ*varA* mutants,

which produce no or reduced amounts of andrimid (Chapter 2), appear to produce hemolysins and extracellular proteases to the same degree as the wild type. This may suggest that the genes *luxT* and *varA*, while involved in the regulation of andrimid production, are not involved in other QS regulated responses. It is also possible that these mutations affected exoenzyme production to a degree undetectable by the qualitative methods used.

In the assessment of the regulation of andrimid production (chapter 2), a Δ*luxO* mutant surprisingly produced less andrimid than the wild type (it was first expected that this mutant would produce more andrimid than the wild type). This trend was not seen with hemolysin or protease production, as the Δ*luxO* mutant produced the same exoenzymatic activity as OCN008

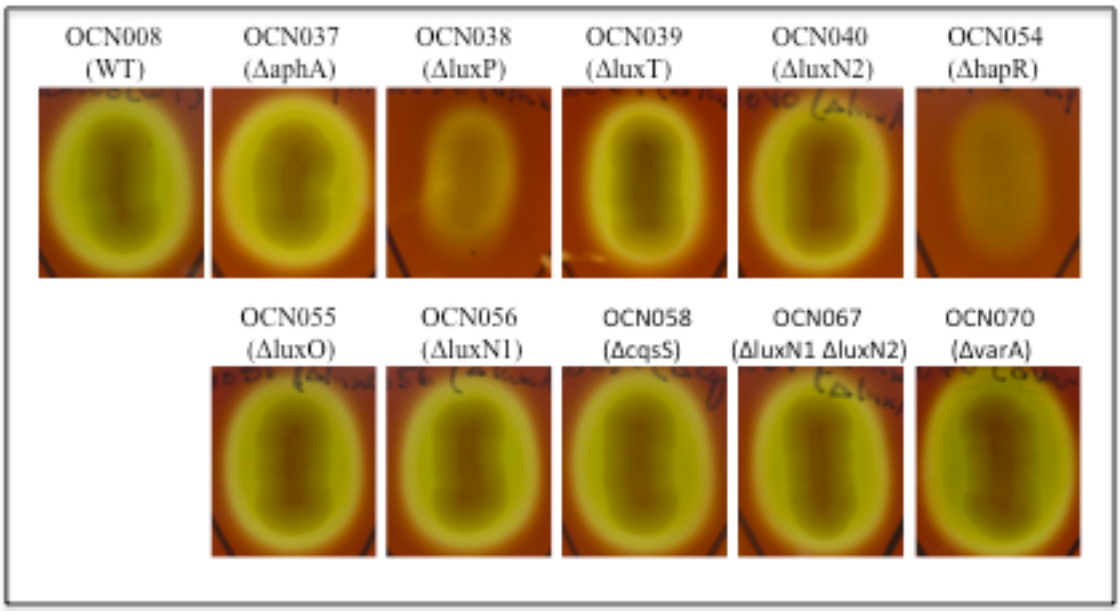

Figure 3.3. Hemolysin production by wild type OCN008 and the QS mutants. Wild type OCN008 displays Beta hemolysis at ~48 hours. All mutants except for OCN038 (Δ*luxP*) and OCN054 (Δ*hapR*) displayed Beta hemolysis in the same manner as the wild-type. OCN038 and OCN054 only display alpha hemolysis over the 96 hours the plates were monitored.

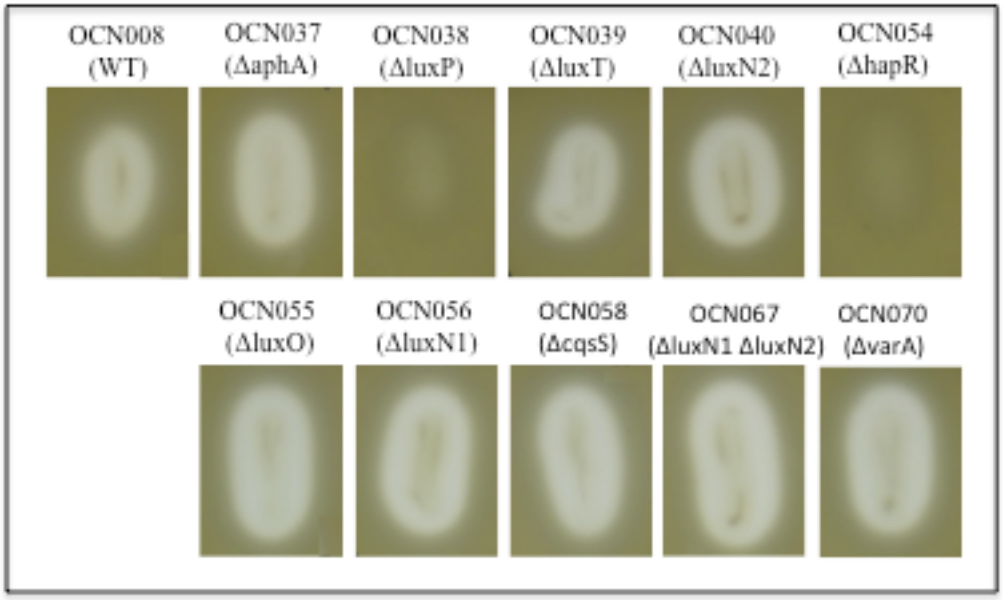

Figure 3.4. Exoprotease production by wild type OCN008 and QS mutants. Wild type OCN008 shows clearing on skim milk agar after ~24 hours. All mutants except for OCN038 (Δ*luxP*) and OCN054 (Δ*hapR*) behaved in the same manner as the wild-type. OCN038 and OCN054 never showed clearing of the skim milk agar over the 96 hours the plates were monitored.

on the BAPs and SMAs. However, the single amino acid mutation from a glutamic acid (D) to aspartic acid (E) at position 47 resulted in reduced hemolytic and proteolytic activity, at levels similar to the Δ*hapR* mutant, demonstrating that *luxO* is involved in the regulation of QS products. This mutation, in *V. cholerae* and *V. harveyi*, causes LuxO to assume a conformation that mimics the LCD form of LuxO, effectively locking the cell into LCD behavior[158]. Since this mutation causes behavior similar to that of the hapR mutant, it is highly suggestive that LuxO's role in QS in OCN008 is similar to that of *V. harveyi* and *V. cholerae,* in that inactivation of LuxO's transcriptional activator activity is critical to activating QS. This data is in agreement with the andrimid findings in that HapR appears to be the HCD master regulator of QS in OCN008 and relies on activation from LuxO, primarily through the LuxPQ/S histidine kinase / synthase QS system.

Experimentation with lipase activity showed that, although OCN008 is lipase positive, lipase production is not under regulation by QS.

QS negatively regulated biofilm formation in OCN008

Biofilm formation is associated with virulence in many pathogens. In *V. cholerae,* biofilm formation is negatively regulated by QS and is critical to virulence. Biofilm formation in OCN008 was assessed using the method developed by O'Toole, et al, using 96 well microplates. Overall, biofilm formation was negatively regulated by QS in OCN008. Biofilm formation was measured by the optical density at 550 nm (OD_{550}) of a 96-well plate as described in the materials and methods. Deletion of *hapR* had the greatest effect of biofilm formation, causing a large increase. Deletion of *luxP* and the *luxOD47E* mutation also caused an increase in biofilm formation, but to a lesser extent. All other mutants formed biofilms comparable to the wild type (figure 3.5).

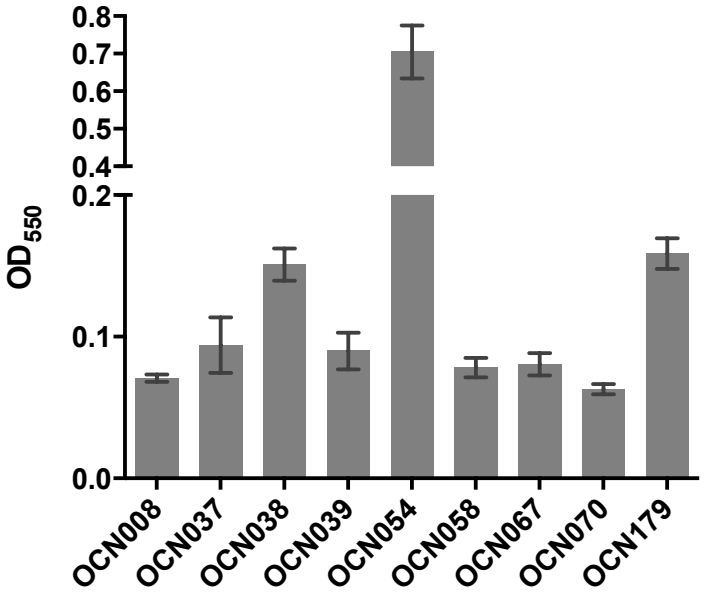

Figure 3.5. Biofilm formation by wild type OCN008 and QS mutants. Biofilm formation was quantified using optical density at 500 nm (OD_{550}). Deletion of *hapR* had the greatest effect of biofilm formation, causing a large increase. Deletion of *luxP* and the *luxOD47E* mutation also caused an increase in biofilm formation, but to a lesser degree.

QS is involved in the infection of M. capitata by OCN008.

To determine if QS is involved in the infection of *Montipora capitata* by OCN008, *in vitro* infection assays were conducted with QS mutants. Due to the sensitive nature of coral collection and the large number of coral fragments needed to run an infection assay, only mutants that displayed a down-regulation of the QS response were tested for infection. In laboratory infection trials, wild-type OCN008 infects fragments of the coral *M. capitata* at a rate of about 80%-90%[99]. To test if the QS genes shown here to be important for hemolysin, protease, biofilm or antimicrobial activity are important for infection of coral, infection trials were run with the Δ*luxP*, Δ*luxT*, Δ*hapR* and Δ*varA* strains. In addition, the Δ*aphA* strain was selected since AphA is the LCD master regulator and is known to regulate many virulence factors, including the toxin co-regulated pilus (*tcp*), in *Vibrio cholera*[169]. The wild-type strain caused tissue lysis in 86% of inoculated fragments (n=21) with an incubation period ranging from 1 to 7 days. In contrast, the Δ*aphA*, Δ*luxP*, Δ*luxT*, and Δ*hapR* strains all showed a significant attenuation of infectivity relative to that of the wild type, causing tissue lysis in 25% (Mantel-Cox Test, n=12, $p<0.0001$), 33% (Mantel-Cox Test, n=12, $p<0.0001$), 33% (Mantel-Cox Test, n=12, $p<0.0001$), and 11% (Mantel-Cox Test, n=9, $p<0.0001$) of the fragments inoculated, respectively. The Δ*varA* mutant also showed attenuated infectivity: the wild type caused tissue lysis in 57% of the coral fragments inoculated, and the Δ*varA* mutant did not cause any signs of disease in any (0%) inoculated fragments (Mantel-Cox Test, n=7, $p=0.022$) (figure 3.6). The incubation period was similar to that of wild type for all fragments infected by the mutant strains, suggesting that when these strains do infect, infection proceeds at a rate similar to that of the wild type. These data are indicative that QS is involved, but not required, for infection of healthy *M. capitata*.

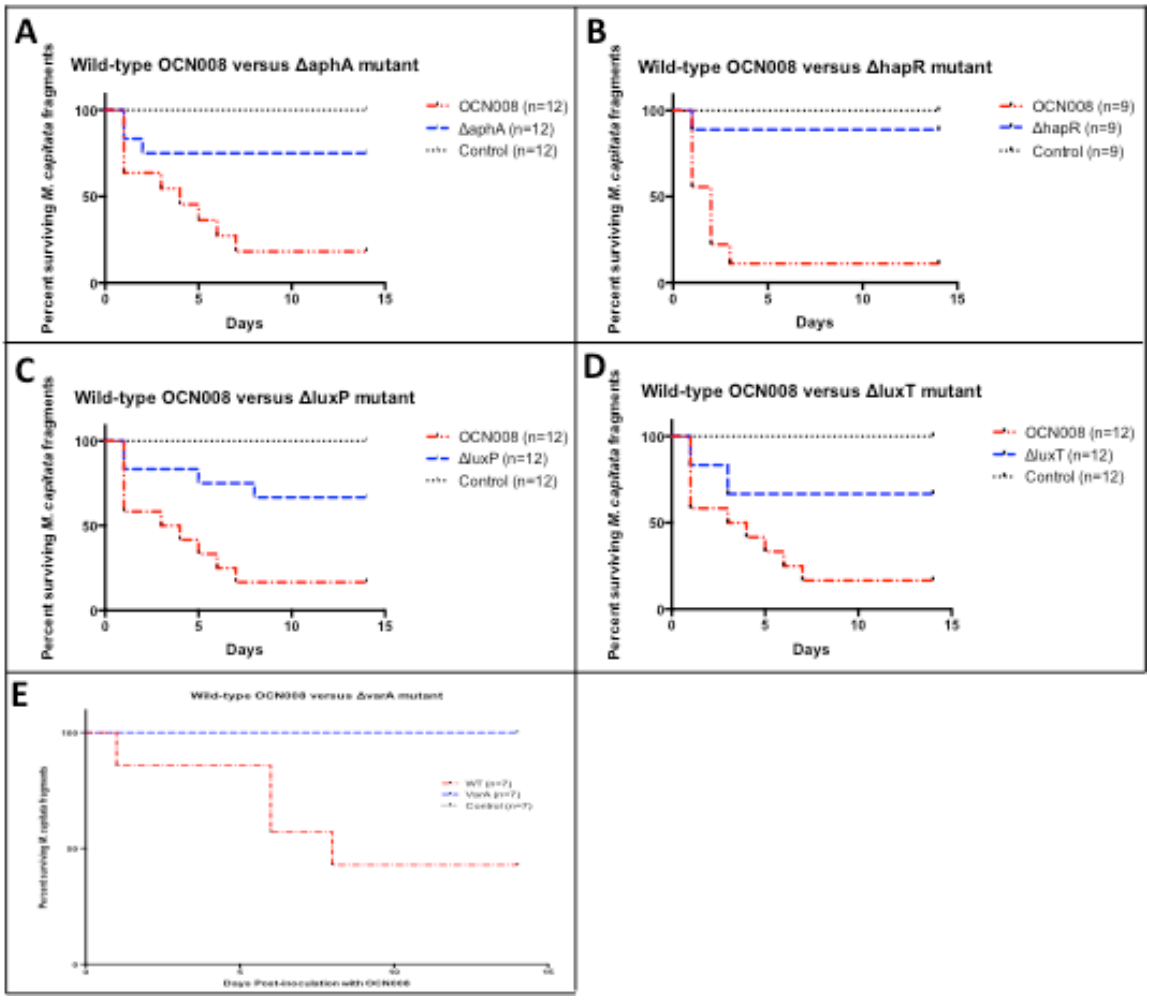

Figure 3.6. Kaplan-Meier survival curves of M. capitata fragments inoculated with wild type OCN008 and the QS mutants OCN037, OCN038, OCN039, OCN054 and OCN070.

DISCUSSION

In this work three virulence-associated, QS regulated phenotypes were identified. The work was inspired by the discovery that the antibiotic andrimid, a novel virulence factor, is regulated by QS in OCN008. Extracellular hemolysins and proteases are positively regulated, and biofilm formation appears to be negatively regulated by QS in OCN008. The HCD master regulator HapR and LuxP of the LuxPQ/S QS detection circuit were found to be required for β-hemolysin and high levels of extracellular protease production. Biofilm production was also

affected by the deletion of *hapR* and *luxP*. Deletion of *hapR* greatly increased biofilm formation, which mirrors *V. cholerae* isolates that contain frame-shift mutations in *hapR*. Until this assay, the Δ*luxP* and *luxOD47E* mutants had behaved nearly identically to the Δ*hapR* mutant. This was somewhat expected as these mutations prevent HapR activation. One key difference between the three mutants, however, is that, in the case of the hapR mutant, the QS sensing apparati are functional; specifically, at HCD LuxO loses phosphorylation transcriptional activation activity leading to abrogation of QS regulatory RNA (qrrs) transcription. In the Δ*luxP* and *luxOD47E* mutants, presumably LuxO either remains phosphorylated or maintains a form that mimics the transcriptionally active form and transcription of qrrs continues throughout HCD. The difference in the level of qrrs present at HCD between the hapR, luxP and luxOD47E mutants may explain the difference seen in biofilm formation not seen in hemolysin and protease production, which rely on the presence of HapR.

Surprisingly, the Δ*luxT* and Δ*varA* mutations that knocked out andrimid production did not have the same effect on hemolysin, protease, or biofilm production and behaved like wild type OCN008. Both LuxT and VarA are little known players in QS, with only a handful of publications mentioning LuxT. VarA has only been mentioned in the context of *V. cholerae* QS and the synthase / autoinducer have yet to be identified. In addition, each *Vibrio* QS circuit has inherent differences in both the architecture and output of the system with different regulatory networks intersecting at multiple points, allowing for a highly complex QS response specific to each QS employing organism[80]. This work, as well as the work surrounding andrimid regulation, suggests that HapR actuates the HCD QS response in OCN008 through the LuxPQ/S system and LuxO, and that other HCD behaviors exist that require more than HapR alone. This can further be justified by 1) andrimid production occurs in only a select few *Vibrio* strains and

thus is not a canonical QS product such as hemolysins and proteases, and 2) the andrimid operon is thought to have been acquired recently through horizontal gene transfer and 3) a Δ*luxO* mutant (a strain that, theoretically, has elevated levels of HapR at LCD) unexpectedly produced less andrimid than the wild type, inconsistent with many HCD behaviors that increase with HapR levels. Taken together, it's no surprise that andrimid regulation is not as straight forward as the HCD response that has evolved over millions of years that includes hemolysins, proteases, and biofilm architecture.

In these screens the Δ*aphA* mutant was included despite the expectation of negative results. Hemolysin and protease production are traditionally HCD behaviors and, therefore, should be unaffected by deletion of the LCD master regulator – as was seen in these data. However, it is impossible to rule out the possibility that hemolysin and protease production was initiated at a lower cell density than normal in this mutant, but cell density was so low that the effect was not seen, highlighting the benefit afforded to cells that employ QS. A quantitative assay with enough sensitivity could resolve this question. Biofilm formation in *V. cholerae* is inhibited by HapR, which is still present in a Δ*aphA* background[82, 124,165]. This is consistent with the above results as the Δ*hapR* mutant consistently produced the largest biofilms by a significant margin. The fact that the Δ*aphA* mutant did not show decreased biofilm formation suggests that positive regulation of biofilm formation is AphA independent in OCN008.

The contribution to virulence provided by each gene was assessed. HapR, unsurprisingly, is highly important for OCN008 virulence in a laboratory setting. The HapR regulon of *V. harveyi* (HCD response) is larger than 500 genes[90]. Thus, it is likely that HapR regulates a similarly large regulon in OCN008. A successful infection requires sequential expression of many virulence factors in a time-dependent fashion, starting with genes involved

with chemotaxis and attachment, to the injection of toxins or invasion of host cells, to genes expressed for nutrient scavenging from lysed tissues[118,170,171]. Thus, the inability to initiate such a large response can easily explain the attenuated virulence displayed by Δ*hapR* mutant. Interestingly, a similar reduction in virulence was seen between the Δ*luxP* and Δ*luxT* mutants. Both mutants do not produce andrimid, but only the Δ*luxP* mutant was different than the wild type in other assays. This may suggest that extracellular hemolysin and protease production are not necessary for the initiation of infection. This is consistent with the thought that, often, these enzymes are involved in nutrient scavenging post-infection. The Δ*aphA* mutant was screened for virulence due to its involvement in the virulence of *V. cholerae.* Similar to *V. cholerae*, AphA appears to be involved in virulence in OCN008, as is HapR. Finally, the Δ*varA* mutant also showed a trend of attenuated virulence. Further study on the infectivity of the Δ*varA* mutant was desired, but an unfortunate outbreak of *Montipora* flat worms yielded too few *M. capitata* fragments to increase the *n* value[172]. Altogether, every mutant deficient in andrimid production showed attenuated virulence, in agreement with the findings that an andrimid operon mutant displays attenuated virulence[99].

This work, which identifies a singular QS pathway as the primary means of HCD master regulator HapR activation, sheds an interesting light onto the infection dynamics between OCN008 and *M. capitata*, in addition to representing the first direct link between QS and pathogenesis in the field of coral disease. The LuxPQ/S pathway, which produces and recognizes the boronated AI-2 molecule, is the most universal QS pathway found in *Vibrios* and bacteria in general[139, 141,173]. If OCN008 relies (mostly) solely on increased AI-2 concentration for QS activation (as this data would suggest), it is possible to have activation of QS without a quorum of *self*,, but, rather, simply a quorum of AI-2 producing bacteria. QS appears to activate

andrimid production, which is hypothesized to limit the growth of competing bacteria and facilitate OCN008 proliferation. It is believed that the mucus layer of corals normally possesses between 10^5 to 10^6 CFU bacteria/mL of mucus, and that these 'normal flora' play an important role of occupying niches of the coral, preventing infections via competitive exclusion[101,102,104,174]. If cells are evenly distributed, this cell density would likely be too low to activate QS under normal conditions, but if an event occurs that greatly increases the local cell density enough (such as sewage runoff, or nutrification that spurs the blooms of fast-growing heterotrophs) QS would be activated and infection could ensue. Conversely, locally elevated concentrations of bacteria could facilitate QS activation. Increasing efforts to prevent and understand bacterial load associated with sewage spills could help prevent large coral disease outbreaks such as the aMWS outbreaks of 2010 and 2012. Both outbreaks, incidentally, occurred following heavy rain events and were the most prevalent and abundant in areas of the Kaneohe Bay most greatly affected by human development[23]. While data on the shifts in microbial populations and loads associated with the events preceding these outbreaks is limited, it is not without reason that the increased runoff caused spikes in bacterial populations, either through direct deposit of terrestrially associated bacteria or the deposit of large volumes of nutrients that are known to cause blooms in certain strains of marine bacteria. Regardless, results from work such as the work described above highlight the importance of understanding the underlying mechanisms behind coral diseases to ultimately prevent or even cure these economically and ecologically damaging diseases.

Table 3.1. Strains used in this study

***Vibrio Coraliillyticus* Strains**	**Relevent Charachteristics ***	**Source or Reference**
OCN008	Wild Type; Ap^R	[61]
OCN037	Δ*aphA;* Ap^R	chapter 2
OCN038	Δ*luxP;* Ap^R	chapter 2
OCN039	Δ*luxT;* Ap^R	chapter 2
OCN040	Δ*luxN1;* Ap^R	chapter 2
OCN054	Δ*hapR;* Ap^R	chapter 2
OCN055	Δ*luxO;* Ap^R	chapter 2
OCN056	Δ*luxN2;* Ap^R	chapter 2
OCN058	Δ*cqsS;* Ap^R	chapter 2
OCN067	Δ*luxN1*Δ*luxN2;* Ap^R	chapter 2
OCN070	Δ*varA;* Ap^R	chapter 2
OCN179	*luxO(D47E);* Ap^R	chapter 2
Marine Bacterial Strains		
OCN004	*Alteramonas* sp. found on healthy *M. captitata*	[24]

*Ap^R – Ampicillin resistance

CHAPTER FOUR: ASSESSMENT OF HAPR PROTEIN LEVELS IN OCN008

INTRODUCTION

Quorum sensing (QS) in bacteria is defined as coordinated gene expression from groups of cells in a cell-density dependent fashion[80,81,126]. First discovered in the genus *Vibrio*, QS has since been discovered in many bacteria, gram-negative and gram-positive. At its heart, QS relies on the presence or absence of two regulators, a low cell density (LCD) and a high cell density (HCD) master regulator[90]. A representative diagram of QS in *Vibrio cholerae and V. harveyi* is shown in figure 4.1. In *V. cholerae,* a reference genome used to help identify possible QS genes, these regulators are known as AphA and HapR, respectively[124]. The regulator present at any given time is dependent upon the ratio of occupied to unoccupied histidine kinase receptors. Each histidine kinase receptor binds a specific molecule, called an autoinducer (AI). Autoinducers are synthesized at constant rates by cells and excreted into the environment. This is the how cells can detect population sizes. At LCD very few AIs are present, and at HCD AIs become abundant. At LCD, the unoccupied histidine kinase receptors autophosphorylate, creating a phosphate flow that results in the phosphorylation of LuxO (through the phosphor-relay protein LuxU)[175]. LuxO, in its phosphorylated form, is an active transcriptional activator that induces the transcription of the quorum sensing regulatory RNAs (qrrs)[88, 159,176]. These qrrs determine which master regulator is present – they aid in the expression of AphA and also bind the HapR mRNA, leading to its degradation[89]. At HCD, most of the histidine kinase receptors are occupied and the phosphate flow reverses, ultimately yielding the inactive, un-phosphorylated LuxO. This form of LuxO is unable to induce qrr transcription, AphA is no longer expressed, and HapR becomes more abundant as its mRNA is no longer degraded.

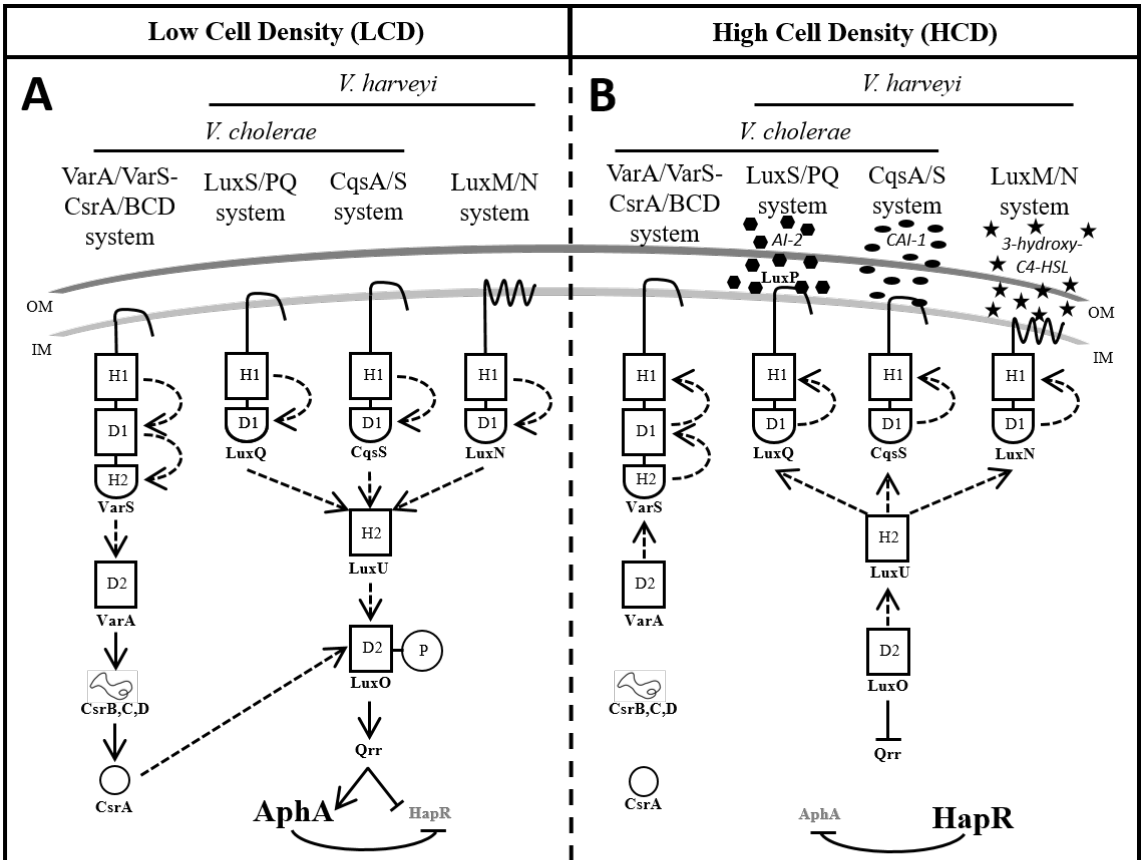

Figure 4.1. Quorum Sensing in Vibrio cholerae and Vibrio harveyi. Each bacterium employs three QS systems, two of which are shared between the two species. Horizontal lines above the system indicate which pathways exist in each organism. Dashed lines indicate the flow of phosphorylation. **A)** Low Cell Density (LCD). At LCD, extracellular autoinducer (hexagons, ovals, and stars) concentration is low and histidine kinase receptors are unbound. This induces a phospho-relay pathway ending with a phosphorylated, transcriptionally active LuxO. The phosphorylated LuxO then in-directly induces expression of the LCD master regulator AphA and repression of the HCD master regulator HapR. **B)** High Cell Density (HCD). At HCD, extracellular autoinducer concentration is significant enough to bind their cognate histidine kinase receptors, inducing a reverse in the phospho-flow. This results in a de-phosphorylated LuxO that becomes transcriptionally inactive. AphA expression is then repressed and HapR becomes the dominant regulator of the system. Some classical QS behaviors for each organism at each state are listed below the diagram.

Vibrio coralliilyticus strain OCN008 was recently discovered as an etiological agent of the coral disease Acute *Montipora* White Syndrome (aMWS)[61]. This tissue loss disease represents a threat to the Hawaiian reef building coral *Montipora capitata,* particularly colonies found in Kaneohe Bay, Oahu[23]. This pathogen is unique in that it produces the antibiotic andrimid as a virulence factor – the only known pathogen to do so[99]. Previous work determined that andrimid was under regulation by QS in OCN008 (chapter 2). Additionally, it was found that aspects of QS regulon are involved in virulence, as the deletion of either the LCD or the HCD master regulator of QS attenuated virulence (chapter 3). This discovery was the first direct link between a genetic regulatory network and pathogen virulence in the field of coral disease. As such, QS represents a promising target for potential preventative or curative strategies. Such strategies are desperately needed as coral disease worldwide continues to worsen and have the potential to cause coral mortality at rates that far exceed rates of coral growth and settlement.

HapR, the high cell density master regulator of the QS regulon, is post transcriptionally regulated[82,177]. The fine interplay between the levels of AphA and HapR determine the QS response[82,90]. In the previous chapters, 4 extracellular products (andrimid, hemolysins, proteases and biofilms) have been screened as a proxy for the QS response in OCN008. Given the large QS regulons of other *Vibrios*, it is safe to assume that more genes under regulation of QS in OCN008. In *Vibrio harveyi* it was found that the high cell density master regulator of QS regulates over 500 genes directly. Due to the large nature of the QS regulon and wide array of QS responses controlled by highly specific levels of HapR, it is possible that genes thought to be uninvolved with QS based on the previous screens actually do contribute to QS in OCN008 by altering HapR levels in a way that was undetectable in the previous work. To test for this, direct levels of HapR were targeted for measurement by qualitative western blots[178]. The chromosomal

hapR has been tagged with a C-terminal 6-histidine tag which will allow for detection with an anti-his antibody[179]. This chromosomal mutation is able to compliment a HapR deletion, making it suitable for study. Initially, HapR levels of the tagged wild type were measured at different points over a 24-hour growth period. The goal of this work was to more directly ask if genes homologues to QS genes found in other *Vibrios* were involved in QS in OCN008. By doing so, the QS network of OCN008 will be established, providing more data for comparison in field of QS.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All vibrio strains were grown in glycerol artificial seawater (GASW) medium as previously described, except Rila Salts were substituted for Instant Ocean® salt mix (Spectrum Brands Inc., Atlanta, GA) unless otherwise noted[121]. *E. coli* strains were grown in LB supplemented with 0.3M diaminopimelic acid (DAP) or deoxythymidine (DT) as needed. For all solid media, 15 g L^{-1} was added prior to autoclaving. Strains and plasmids used in this study are listed in table 4.1. Conjugation media was supplemented with 1% D-glucose (DEX) and 0.3M DAP and DT. Thiosulfate citrate bile salts sucrose (TCBS) medium (BD, Franklin Lakes, NJ.) supplemented with 15 μg L^{-1} gentamicin (Gm15) was used for selection of single recombinants. Single recombinants were maintained on GASW medium supplemented with 1% D-glucose, 100 μg L^{-1} spectinomycin (Sp100) and 50 μg L^{-1} streptomycin (Sm50). Selection of double recombinants was achieved using GASW medium supplemented with 0.2% arabinose (ARA).

Plasmid and Strain Construction

All strains, plasmids and primers used in this study are listed in table 4.1. Strains carrying the HapR-LacZ and his-tagged HapR constructs were constructed using allelic replacement as previously described using the suicide vectors pAHB185 and pAHB191, respectively[122,180]. Briefly, DT auxotrophic *E. coli* strain π3813 carrying the self-transmissible plasmid pRK2013, DAP auxotrophic *E. coli* strain β3914 carrying a suicide vector, and *Vibrio* strains carrying chromosomal QS gene deletions (described in chapter 2) were grown overnight, diluted 1:1000 in fresh media, and grown to an OD_{600} of ~0.5. Cultures were then washed three times each, and combined into a new tube in the ratio of 10:10:1 *E.coli : E.coli : Vibrio*. Fifty μL of the resulting mixture was the pipetted into a spot on a GASW-DEX plate. Cells were allowed to conjugate overnight, and the following morning conjugation spots were collected and washed three times. Selection for single recombinants was achieved by plating the washed conjugation spot onto TCBS-Cm15 plates. Resulting colonies were then streaked onto GASW-DEX-Sp100-Sm50 plates for single recombinant maintenance. Double recombination was encouraged by growing single recombinant colonies in GASW-DEX liquid medium without antibiotics. Cultures were allowed to grow overnight, washed three times, then plated onto GASW-ARA plates for selection of double recombinant. Double recombinants were screened for the LacZ or his-tagged HapR via PCR amplification of the HapR locus using outside primers.

Plasmid pAHB185 is a suicide vector based on pSW4426T used to add the coding region of LacZ to the 3' end of the coding region of *hapR* as a translational fusion. Three regions of DNA were amplified for a three-part overlap extension: 1) ~800 bp upstream of *hapR* and the *hapR* coding region were amplified using the primer pair hapR-up-EcoRI-F and hapR-lacZ(1)-R, 2) LacZ coding region was amplified from the plasmid pUC18-mini-Tn7-gm-lacZ[181] using the primer pair hapR-lacZ(2)-F and hapR-lacZ(2)-R, and 3) ~800 bp downstream of *hapR* was amplified using the primer pair hapR-lacZ(3)-F and hapR-dn-*EcoRI*-R. The resulting three fragments were

fused together via three-part overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+ (Stratagene). The HapR-LacZ construct was then cloned as an *Eco*RI / *Xba*I fragment into the *Eco*RI / *Spe*I sites on pSW4426T.

Plasmid pAHB191 is a suicide vector based on pSW4426T used to add 18 nucleotides to the 3' end of the coding region of *hapR*. The CDS and regions up- and downstream of *hapR* were amplified by PCR from chromosomal DNA with the primer pairs hapR-up-EcoRI-F and HapR-his-up-R, and HapR-his-dn-F and hapR-dn-EcoRI-R, respectively. The up- and downstream fragments were fused together by overlap extension PCR and cloned into the *Sma*I site of pBlueScript SK+. The HapR-his construct was then cloned as an *Eco*RI fragment into the *Eco*RI site on pSW4426T.

Beta-galactosidase assays

Beta-galactosidase assays were performed with OCN008 and OCN160. WT OCN008 and OCN160 (HapR-lacZ) were grown in GASW to the indicated OD_{600}, incubated on ice for >30 minutes, pelleted via centrifugation at 10,000 x *g* (at 4°C) for 10 minutes, and re-suspended in 1 mL of Z-buffer. Beta-galactosidase assays and Miller Unit conversions were conducted as previously described[182]. Data was analyzed using GraphPad Prism (version 6 for MacOSX), GraphPad Software, La Jolla California USA, www.graphpad.com).

Western Blots

Strains used for western blotting were grown in 50 mL GASW to the indicated OD_{600} and cells were pelleted via centrifugation at 10,000 x *g* for 10 minutes at 4°C, and pellets were frozen at -80°C until use. Before use, samples were removed from the -80°C freezer and thawed on ice, re-suspended in 5 mL lysis buffer (70 mM NaHCO3, 30 mM Na2CO3, pH = 9.6), and lysed via

wand sonication on ice using 15 second pulses for a total of 2 minutes, with 30 seconds between each pulse. Cell lysate was then centrifuged in 2mL tubes at 16,000 x *g* to separate insoluble material, and 1 mL of lysate was removed off the top for quantification and western blots. Protein concentrations were calculated using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA). Samples were all diluted to 10 mg / mL after quantification, and 210 μg of protein were loaded onto each polyacrylamide gel. Protein gels were 12% bis-tris gels, and run at 200V for 45 minutes. Blotting was achieved using a Trans-blot® semi-dry blotting cell (BioRad Laboratories, Hercules, CA) at 10V for 30 minutes. Chemiluminescent detection was achieved using Western Breeze Chemiluminescent Kit (ThermoFisher, Waltham, MA). All images were captured using a GeneGnome unit and Syngene software (Syngene, Bangalore, India).

RESULTS

HapR protein levels increase with cell density

HapR levels were measured throughout growth using two methods: western blots with the his-tagged HapR described above, and β-galactosidase assays with a strain carrying a chromosomal HapR-lacZ translational fusion. The HapR-lacZ strain was constructed and screened first (figure 4.2). HapR concentrations increased with cell density, as typically seen in *Vibrios*[177]. LacZ activity was measured in Miller Units, a per-cell unit, so the increase in HapR seen is not simply because there are more cells at higher ODs, but rather the amount of HapR per cell is increasing. Unfortunately, it was discovered that the HapR-lacZ construct was unable to compliment a HapR mutant, and thus the hexa-his construct was made.

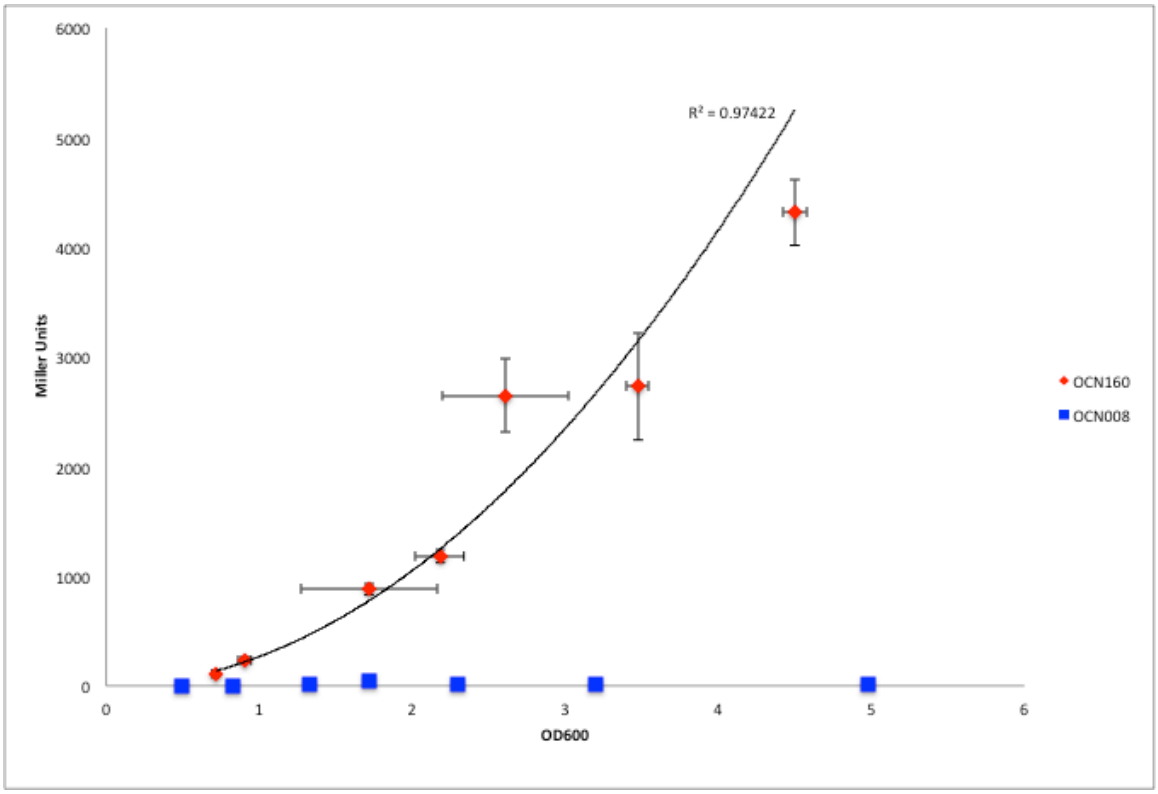

Figure 4.2. HapR-lacZ activity in WT OCN008 and OCN160 (HapR-lacZ) over a 24-hour growth curve. LacZ activity is shown in Miller Units, a per cell quantitative measure. The experiment was done in biological and technical triplicate. Horizontal error bars represent standard deviation of the OD_{600}s between the biological replicates, and vertical error bars represent standard deviation of lacZ activity between biological and technical replicates. Black line represents the best-fit line, $R^2 = 0.9742$.

Western blot analysis of the his-tagged HapR protein at different optical densities (ODs - measured at 600 nm) confirmed the observation seen with the LacZ reporter (figure 4.3). Samples of OCN161 (the his-tagged OCN008) were taken at OD_{600}s of 0.4, 1.25, 1.5, 2.3, 2.8, 3.4, 4.1, and 4.8 for western blotting. Equal amounts of protein (~210 µg) from each time point was loaded as a means of standardization. Protein bands appeared between the 20 kDa and 25 kDa bands of the ladder. This is as expected as the his-tagged HapR protein consists of 166 amino acids and is predicted to be 21.55 kDa. Qualitatively, the band intensity increased with cell density, with the most intense bands coming from the sample taken at OD_{600} 4.8. Wild type

OCN008 (expresses HapR without the his-tag) was used as a negative control, and no banding was observed at any point.

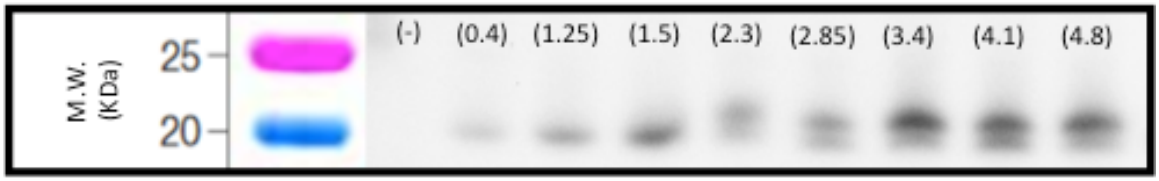

Figure 4.3. HapR levels at different cell densities as measured via western blots. OCN008 serves as the negative control (left lane). OCN161 cells were harvested at the OD_{600} shown above the lanes. The positive control is a sample from OCN161 initially used to show the specificity of the anti-tetra-histidine antibody, and thus is known to be positive for HapR.

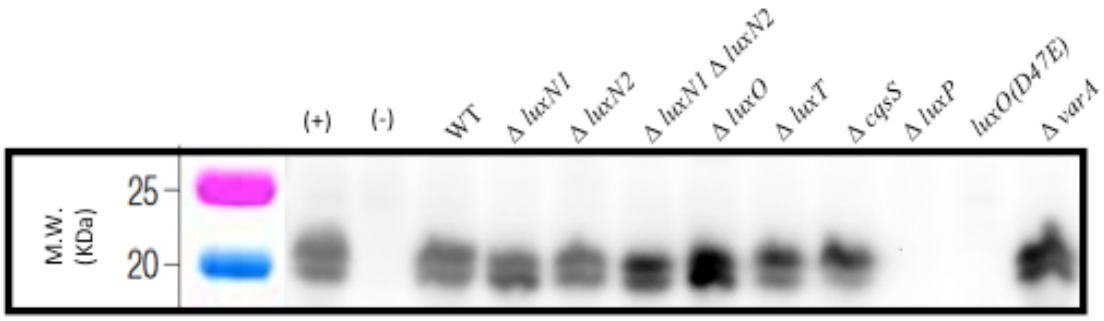

Figure 4.4. HapR production in WTOCN008 and quorum sensing mutants. Samples were taken at two OD600s: one taken in mid-to-late exponential growth phase (all samples were taken at OD_{600} between 2 and 2.5), and the other taken from overnight cultures (late stationary phase) at OD_{600} between 3.2 and 4. The image shown is the best representative image from three independent trials.

LuxP and a functional LuxO are required for HapR protein expression

To assess HapR levels mutants lacking genes homologous to known QS pathways, the his-tagged HapR was inserted chromosomally into strains OCN037 (Δ*aphA*), OCN038 (Δ*luxP*), OCN039 (Δ*luxT*), OCN040 (Δ*luxN1*), OCN055 (Δ*luxO*), OCN056 (Δ*luxN2*), OCN058 (Δ*cqsS*), OCN067 (Δ*luxN1*Δ*luxN2*) and OCN070 (Δ*varS*). Additionally, OCN179, described earlier in this dissertation as carrying a point mutation in LuxO (LuxO[D47E]), was initially constructed in the his-tagged HapR background and was also screened. Cells were harvested at two time-points: 1) a late exponential / pre-stationary phase time-point (ODs between 2.0 and 2.8), and 2) a stationary phase time point (OD_{600}s between 3.2 and 4). All mutants except for Δ*luxP* and *luxO(D47E)* produced detectable HapR at the same level as the WT at both time points (figure 4.4). No bands were observed at any point in all samples from taken from the Δ*luxP* and *luxO(D47E)* mutants, demonstrating that LuxP and a functional LuxO are required for HapR protein expression.

DISCUSSION

This work was undertaken in an attempt to better understand the specific details of quorum sensing in *Vibrio coralliilyticus* strain OCN008. In the previous chapters, it was established that andrimid, hemolysin, protease, and biofilm production were under regulation of QS in OCN008. It was demonstrated that HapR (ERB64458) is the master regulator of QS in OCN008, and that the LuxPQ/S pathway is the primary pathway regulating HapR expression. In addition, QS was shown to be involved in the infection of the coral *Montipora capitata* by OCN008. However, in all previous studies, QS activity was measured via the detection of extracellular QS products. More specifically, the statement 'LuxPQ/S is the primary pathway

involved in QS activation in OCN008', has ultimately meant that the LuxPQ/S is the primary QS system involved in andrimid, hemolysin, protease and biofilm production. Given the large regulon of HapR, defining the requirement for a gene to be involved in QS based on that gene's effect on only a small handful of QS outputs (4 out of potentially 500+) is incomplete. Any synthase / histidine-kinase-receptor pair that affects HapR levels would classically considered as involved in QS. The methods used in chapters two and three would only have classified a gene as involved in QS if the deletion of that gene had affected the level of HapR significantly enough to cause a change in the expression of the extracellular products, rather than that gene's effect on HapR expression.

In order to truly examine if genes dubbed not involved in QS based on results seen in chapters two and three, levels of HapR protein were directly measured via β- galactosidase activity and western blots in strains carrying chromosomal HapR-LacZ and HapR-histidine$_6$ tags, respectively. Consistent with other *Vibrio* QS systems, cellular levels of HapR increased with cell density. This was demonstrated initially with β-galactosidase assays using a chromosomal *hapR-lacZ* translational fusion. It should be mentioned that a HapR-LacZ fusion failed to complement a *hapR* deletion. Its no surprise that the act of linking a small transcriptional regulator (such as HapR) with a large protein like LacZ renders the tagged protein non-functional. The primary regulation of the expression HapR, however, has been shown to occur at the RNA level, circumventing the need for a functional HapR protein to evaluate HapR regulation, barring the event that a functional HapR regulates itself. Since it is not uncommon for transcriptional regulators to self-regulate, it seemed appropriate to evaluate HapR protein levels with a tag that did not interfere with the functionality of the protein. This was achieved via western blot assessment of a chromosomal HapR-histidine$_6$ tag at different growth phases.

In general, results from western blot experiments were consistent with the trend seen in the β- galactosidase experiments. Interestingly, HapR was detectable at every growth phases tested. Andrimid production becomes detectable only at densities of OD_{600} 2.8 – 3, suggesting that either 1) the andrimid production is induced only at high HapR concentrations, or 2) the detection of andrimid production can only be achieved *in vitro* when OCN008 is grown to high cell densities. Since andrimid production is a novel virulence factor, and QS was shown to be involved in the virulence of OCN008 earlier in this dissertation, each scenario presents interesting implications in regards to aMWS. In the scenario that andrimid production only occurs at high cell densities relative to what is typically seen in the environment, it would suggest that large inputs of AI-2 molecules, AI-2 producing bacteria, or nutrients enabling OCN008-like cells to proliferate to higher than environmentally normal cell densities for QS to activate. On the other hand, if andrimid production is induced at cell densities lower than detectable using the methods in this dissertation, it becomes possible that relatively small increases in cell densities found residing in coral mucus and seawater (10^5-10^6 and 10^4-10^6 CFU / mL[101, 103,142]), respectively) could activate andrimid production, QS through HapR and initiate and infection.

HapR production was detectable via western blot in all mutants except Δ*luxP* and *luxO(D47E)*, confirming results shown earlier in this dissertation that indicate the LuxPQ/S system as the primary pathway regulating HapR, and therefore QS, in OCN008. While the western blot transfers could be improved, the general trend was consistent in all replications. In chapters two and three, a Δ*luxT* mutant was incapable of producing andrimid, similar to the QS deficient *ΔhapR* mutant, but did excrete extracellular proteases and hemolysins, unlike the Δ*hapR* mutant. Here, it is shown that the Δ*luxT* mutant produces detectable HapR, indicating

that the Δ*luxT* mutation does not prevent HapR expression, and thus LuxT regulation of andrimid production lies downstream of HapR. The Δ*varA* mutant, which produces less andrimid than OCN008, produces a detectable HapR. This may explain why these two strains were not noticeably different than the wild type in hemolysin, protease and biofilm assays (chapter 3).

Table 4.1. Strains, plasmids, and primers used in this study

Vibrio Coraliillyticus Strains	**Relevent Charachteristics ***	**Source or Reference**
OCN008	Wild Type; Ap^R	[61]
OCN160	HapR-lacZ; Ap^R	This Study
OCN161	OCN008 w/ *hapR-his;* Ap^R	This Study
OCN162	Δ*luxN1-hapR-his;* Ap^R	This Study
OCN163	Δ*luxN2-hapR-his;* Ap^R	This Study
OCN164	Δ*luxN1ΔluxN2-hapR-his;* Ap^R	This Study
OCN165	Δ*luxO-hapR-his;* Ap^R	This Study
OCN166	Δ*luxT-hapR-his;* Ap^R	This Study
OCN167	Δ*cqsS-hapR-his;* Ap^R	This Study
OCN168	Δ*luxP-hapR-his;* Ap^R	This Study
OCN179	*luxO(D47E)-hapR-his;* Ap^R	This Study
OCN216	Δ*varA-hapR-his;* Ap^R	This Study
E. Coli Strains		
β3914	DAP Auxotroph; Km^R	[122]
π3813	Thymidine Auxotroph; Em^R	[122]
Plasmids		
pBlueScript SK+	Cloning vector; Ap^R	Stratagene
pRK2013	Self Transmissible plasmid; Km^R	[147]
pUC18-mini-Tn7-gm-lacZ	Source of LacZ; Ap^R, Gm^R	[181]
pSW4426T	Suicide Vector used to make mutations; Cm^R, Sp^R, Sm^R	[122]
pAHB185	pSW4426T::HapR-LacZ; Cm^R, Sp^R, Sm^R	This study
pAHB191	pSW4426T::HapR-his tag; CmR, SpR, SmR	This study
Primers		

hapR-up-EcoRI-F	ATATAGAATTTCCTCACGACGAGACGGCTTATCAAGCAGCGTAC
hapR-his-up-R	CTAGTGATGGTGATGGTGATGCTTGTAGATGCAAAGCATATCTAG
hapR-his-dn-F	CATCACCATCACCATCACTAGATTAACCAGTGTCATCGTAAAGCC
hapR-dn-EcoRI-R	ATATAGAATTCGACGGTATCTACGTGTCAATGGAAGGCAAG
hapR-outside-F	CTGCGCGTAATCAATACCCACACCAACAAC
hapR-outside-R	GATATCGTTAAAGTTTACACTAAGCGTGTG
hapR-lacZ(1)-R	CGAACCCGAACCCGACGAACCACCCTTGTAGATGCAAAGCATATCTAGGTAG
hapR-lacZ(2)-F	GGTGGTTCGTCGGGTTCGGGTTCGATGACCATGATTACGGATTCACTGGCCG
hapR-lacZ(2)-R	CACTGGTTAATTTATTTTTGACACCAGACCAACTGG
hapR-lacZ(3)-F	GTCAAAAATAAATTAACCAGTGTCATCGTAAAGCCG

*Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Em^R, erythromycin resistance; Gm^R gentamycin resistance; Sp^R, spectinomycin resistance; Sm^R, streptomycin resistance.

CHAPTER FIVE: SUMMARY AND IMPLICATIONS

Coral reefs encompass some of the most diverse and endangered ecosystems on the planet[183,184]. Climate change, human impacts, and increasing occurrences of disease outbreaks all threaten to degrade reefs worldwide[185,186]. Such an outcome has the potential to devastate many tropical communities and economies. It has been estimated that one third of the world's reef building corals face an increased risk of extinction due to climate change and human impacts[13]. Coral disease has become a major contributor to worldwide reef degradation, and outbreak events have been linked repeatedly to human and climate impacts[187,188]. Disease induced reef degradation perhaps is best exemplified across the Caribbean, where losses of *Acropora palmata* and *A. cervicornis* cover in some areas have exceeded 90% due, in large part, to numerous outbreaks of White Band, White Plagues Types I and II, and White Pox diseases[38–40, 66, 93,189]. This disease-wrought reef destruction can be severe. Loss of acroporid cover in the Caribbean eventually led to the classification of *A. palmata* and *A. cervicornis* as endangered species[190]. As climate change continues to stress marine ecosystems, disease outbreaks have become more common and more severe, thus highlighting the need to understand the drivers and mechanisms of these diseases.

The field of coral disease is young, with the first description of a coral disease coming out of the Caribbean in the 1970s[47]. For the following 20-30 years little progress was made, due, in large part, to a lack of research efforts. However, in the past decade the field has begun to burgeon. Numerous coral disease pathogens have been identified within the last ten years and a better understanding of disease presentations has allowed for faster identification and response to specific diseases. Despite significant strides in the identification of etiological agents, data behind the mechanisms of coral disease remains scarce. To date, only three genes have been

demonstrated as virulence factors: the flagellum (*flhH)* was shown to be required for the infection of *Pocillopora damicornis* by *Vibrio coralliilyticus* strain BAA-450, and the genes *mshA* and *toxR* were shown to be involved in the infection of *A. cytherea* by *V. coralliilyticus* strain OCN014[52,191]. Studies such as these are of value because understanding the mechanisms employed by pathogens will be crucial to the development of preventative strategies in the future.

Vibrio coralliilyticus strain OCN008 has recently been demonstrated as an etiological agent of Acute *Montipora* White Syndrome, a progressive tissue loss disease affecting the reef-building coral *Montipora capitata*[23,61]. Strain OCN008 is a particularly interesting pathogen because it is the only pathogen in any disease or infection model (not just coral disease) to date that has been shown to produce an antibiotic, andrimid, as a virulence factor[99]. This discovery led to the following hypothesis on how an aMWS infection could initiate: 1) cells of OCN008 colonize the surface of a coral; 2) andrimid production prevents proliferation of nearby bacteria effectively removing any competitive pressure; 3) OCN008 reproduces rapidly and eventually overtakes the coral. This model, however, is incomplete. In laboratory infection trials coral fragments must be exposed to levels of OCN008 significantly higher than those found under natural conditions. Additionally, OCN008 was initially isolated from healthy coral, and OCN008 has been found in the environment near corals in the absence of disease signs[100]. This suggests that the initiation of infection requires more than simply the presence of OCN008. To gain a better understanding of how OCN008 is capable of inducing aMWS, this work aimed to unravel the quorum sensing systems employed by OCN008 and how they relate to virulence.

Quorum sensing (QS) in bacteria can be defined as the ability to shift gene expression (behavior) in a cell density dependent manner. This phenomenon was first studied for its role in

bioluminescence in *Vibrio fischeri*, a symbiont of the Hawaiian Bobtail squid. Since its discovery, QS has come to be more closely associated with the virulence of different *Vibrios* rather than symbiosis. The most well defined QS circuits exist in *V. cholerae*, famous for human disease, and *V. harveyi*, another bioluminescent bacterium that has recently been described as a shrimp pathogen. QS relies on the production and detection of molecules called auto-inducers (AIs). The extracellular concentration of AIs determines which of two transcriptional regulators are expressed, thereby affecting a change in gene regulation. The protein AphA is known as the low cell density (LCD) master regulator, and HapR is the high cell density (HCD) master regulator. The extracellular AI concentration affects the expression of the master regulators by causing a directional change of a phosphorylation chain mediated by inner-membrane spanning histidine kinase receptors (HKRs). At low cell density, when AI concentration is low, the unbound HKRs auto-phosphorylate, resulting in expression of a set of RNAs known as quorum sensing regulatory RNAs (Qrrs). In the presence of these RNAs, AphA is expressed and HapR is repressed. Once HCD is achieved, AIs bind their cognate HKRs and the phosphorylation flow reverses, leading to repression of the Qrrs, AphA repression and HapR expression. The shift in gene expression between LCD and HCD can be quite large. In *Vibrio harveyi*, AphA independently regulates >120 genes and LuxR (the HapR homologue) regulates >500[90]. Typically, *Vibrios* employ multiple HKRs, each recognizing a distinct AI, that function in parallel to control the direction of the phosphor-flow. Interestingly, in most described pathogenic *Vibrio* QS systems, there exists one or more HKR(s) that recognize molecule(s) produced only by the cells possessing that HKR(s) and an second HKR capable of recognizing a more universally produced AI. In *Vibrios* the latter is AI-2, a furanosyl borate diester, and its

HKR LuxPQ. This system is not limited to *Vibrios*, and is often referred to as the 'inter-species' QS molecule.

This work, which aimed to describe QS in OCN008, provides evidence that OCN008 relies primarily on only one described QS pathway to initiate QS through the HCD HapR (figure 5.1). First, only two classical QS pathways were shown to be involved in andrimid production: the LuxP/QS and the VarS/A pathways, as strains carrying mutations in each pathway were deficient in andrimid production. The LuxPQ/S pathway, however, was the only pathway shown to be required for QS. Deletion of *luxP* yielded a total abrogation of andrimid production, as did deletion of *hapR* (the HCD master regulator) and the *luxO(D47E)* mutation (a situation where HapR expression is constantly repressed). These mutations also attenuated the virulence of the corresponding mutants, providing the first direct evidence of the involvement of QS in coral disease. Additionally, only the *luxP* mutation prevented detection of HapR protein in western blot experiments, demonstrating the requirement of a functional LuxPQ/S system to affect HapR expression. Given that AI-2 is spoken of as the inter-species communication molecule and how universal the *luxS* gene is, it is not unreasonable that OCN0008 could turn on QS in response to a quorum of any AI-2 producing bacteria. This quorum could even be multi-species. This has significant implications in the world of coral disease, where the dynamics between normal coral microbiota and potential pathogens determines if disease onsets.

It has been hypothesized that the bacteria found on the surface layers of coral play important roles in coral health[101]. Most notably, they occupy space on the coral, thereby preventing possible pathogens from gaining access to the coral animal. It has also been demonstrated that mucus layers of coral possess between $10^5 – 10^6$ CFU/mL of mucus. This

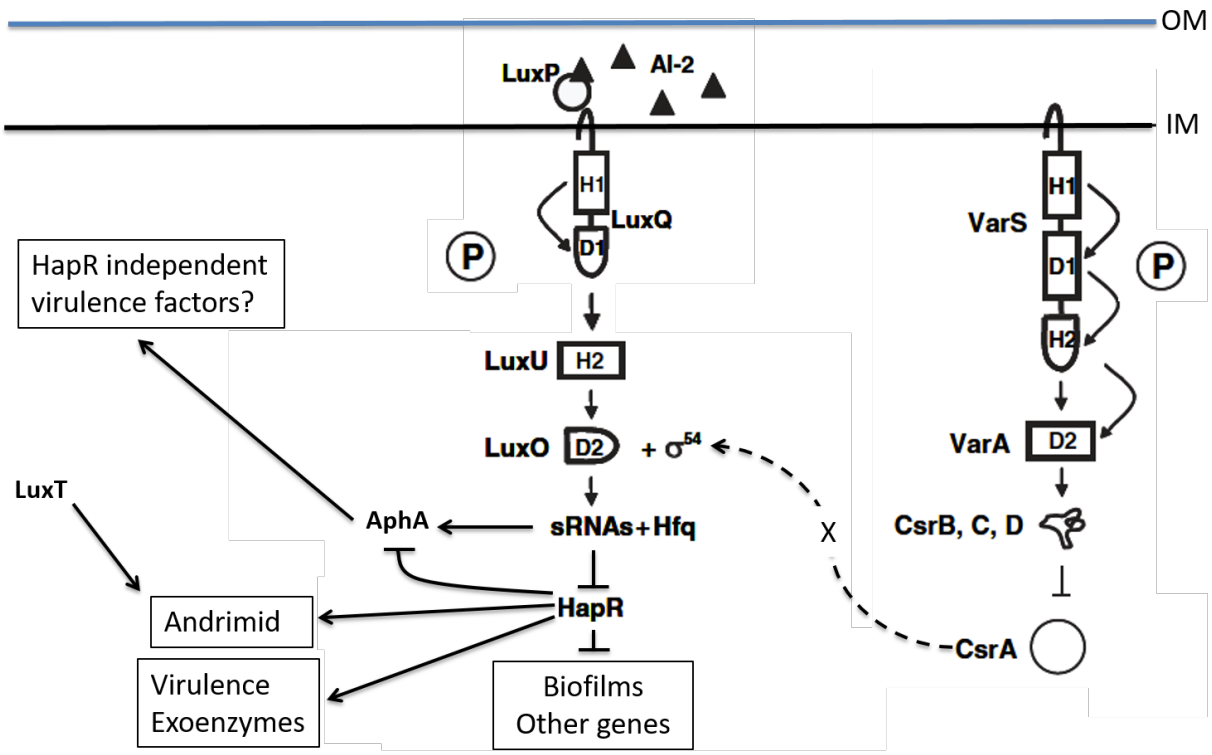

Figure 5.1. Quorum Sensing in Vibrio coralliilyticus strain OCN008. Two quorum sensing circuits are utilized by OCN008: the LuxPQ/S and VarS/A pathways. The LuxPQ/S system is the dominant pathway regulating the activation of the high cell density master regulator HapR. QS, through HapR, negstively regulates biofilm formation, positively regulates extracellular hemolysin and protease production and contributes to virulence during infection of the coral *Montipora capitata.*

number, if bacteria are distributed evenly in the mucous, would be too low to activate quorum sensing and andrimid production in OCN008, which activates at number $>10^{10}$ CFU/mL when grown in Glycerol Artificial Sea Water. This may explain why OCN008-like isolates have been found in disease free areas. However, a perturbation that resulted in an increase in overall bacterial abundance could potentially activate the AI-2 QS pathway and subsequently, andrimid production. Andrimid production, as discussed above would then facilitate proliferation of resistant bacteria such as those similar to OCN008 with disease as the outcome. During the two major outbreaks of aMWS, the disease that initiated this study, effects of the disease were the most pronounced in the southern part of Kaneohe Bay, Oahu[23]. The southern third of Kaneohe

Bay is surrounded by much heavier development than the rest of the bay[34,192,193]. This results in higher levels of land based pollution after rain events. These rain events have been shown to cause nutrification in near shore waters, which can lead to increased bacterial loads[194,195]. Additionally, Kaneohe Bay occasionally experiences sewage spills, events known to spike bacterial loads[196]. Interestingly, the two major outbreak events each occurred following periods of heavy winter rains. It is possible that these heavy rain events caused a significant increase in the concentration of bacteria near corals through nutrification or direct deposit of waste microbes into the environment. This could have caused OCN008-like bacteria in the vicinity to turn on QS and andrimid production, and begin down the road towards infection. This would only be possible because OCN008 relies primarily on the AI-2 pathway to activate the HCD master regulator HapR and, thus, QS.

A coral pathogen reliant exclusively on the LuxPQ/S system has other implications as well. Boron is a component of the AI-2 molecule, and addition of boron to growth media caused an increase in andrimid production, and caused andrimid production to turn on at a lower cell density. Boron is an essential micronutrient in plants widely used in the agricultural industry in fertilizers and pesticides / herbicides (at higher concentrations). As previously mentioned, Kaneohe Bay frequently sees freshwater input from heavy rains and sewage spills. This could potentially activate QS in OCN008, without causing increases in AI-2 producing bacteria, by increasing the concentration of available boron. This would cause levels of AI-2 to increase and allow for QS and andrimid activation at lower cell densities. This could even, perhaps, lower the quorum requirement of OCN008 to levels found frequently on the surface of corals. This would provide an opportunity for infection without greatly altering levels or composition of the normal coral microbiota.

These observations have allowed for the expansion of the hypothesis stated above regarding how an aMWS infection begins. The observation that OCN008-like isolates can readily be isolated near or on healthy coral can be explained by noting that the normal levels of coral microbiota are too low to initiate quorum sensing in OCN008. However, multiple different environmental events could increase this concentration, triggering QS and perhaps infection. This is consistent with the timing of aMWS outbreaks in Kaneohe Bay. Additionally, increases in boron levels have the potential to create a scenario in which OCN008 can activate QS in response to lower cell densities than those typically found under normal circumstances. Given the serious threats facing coral reefs, studies into disease mechanisms have become more and more critical. While much work is left to be done with the *V. coralliilyticus* / *M. capitata* infection model, this work represents a promising starting point to look into QS-based preventative strategies.

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